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RESOLUTION OF INFLAMMATION IN ALZHEIMER'S DISEASE

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**Karolinska
Institutet**

Stockholm 2014

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Published by Karolinska Institutet.

Printed by Åtta.45 Tryckeri AB.

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ISBN 978-91-7549-732-7

Resolution of inflammation in Alzheimer's disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

The thesis will be defended at Hörsalen, Novum, Floor 4, Huddinge,
on Tuesday, December 2nd, 2014, at 9:30

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To my family

Attitude is everything!

ABSTRACT

Inflammation is associated with Alzheimer's disease (AD), a neurodegenerative disorder with no cure up to date. Ample evidence from studies within various disciplines support that inflammation plays a role in AD. Resolution of inflammation is the end stage of inflammation, where the detrimental effects of inflammation are terminated and tissue healing is initiated. Cutting-edge research has demonstrated that resolution of inflammation is controlled by specialized pro-resolving lipid mediators (SPMs). There is emerging evidence for a role of SPMs in various diseases associated with inflammation. One of the SPMs, neuroprotectin D1 (NPD1) was the first to be found in reduced levels in the brain of AD patients. The aims of this thesis were to investigate the resolution status in AD, the mechanisms therein, and the therapeutic potential.

In **Paper I**, we aimed to answer the fundamental question, whether and how the resolution pathway is altered in AD patients. To this end, we analysed cerebrospinal fluid (CSF) and postmortem samples from AD and non-AD patients. We found that in the CSF and hippocampus of AD patients, the levels of one of the SPMs, lipoxin A₄, LXA₄ were lower than that in non-AD groups. This was further confirmed by analysis of a smaller number of hippocampal samples with liquid chromatography - tandem mass spectrometry (LC-MS-MS) technique, which also revealed that another SPM, maresin 1 (MaR1) was reduced in AD hippocampus. Furthermore, the CSF levels of LXA₄, as well as resolvin D1 (RvD1), were positively correlated to mini mental state examination (MMSE) scores. The cellular distribution of two SPM receptors, LXA₄ receptor/formyl peptide receptor 2 (ALX/FPR2) and chemerin receptor 23 (ChemR23) were also described in the human brain. We then investigated if the abnormal resolution may play a role in the pathogenesis of AD. For this purpose, in **Paper II**, we used a senescence-accelerated mouse model, SAMP8 mice, to study the balance between inflammation and resolution during an abnormal aging progress, since the primary risk of AD is aging. We found that inflammation in SAMP8 mice increased with age, and was higher than that in age-matched control mice. However, the resolution markers LXA₄ and RvD1 remained unchanged upon age in SAMP8 mice, or equal to the levels in control mice. Thus, SAMP8 mice appeared to have an unresponsive resolution during the abnormal aging. We also found that enzymes involved in the synthesis of SPMs were abnormally regulated during aging in SAMP8 mice, and that this was related to amyloid β (A β) and tau pathology. In **Paper III**, we analysed materials from a double-blind, randomized, placebo-controlled clinical trial, where n-3 fatty acids or placebo were orally supplemented to AD patients for 6 months. Peripheral blood mononuclear cells (PBMCs) were obtained before and after the trial, and incubated with A β *ex vivo*. We found that there was reduced production of LXA₄ and RvD1 by the A β -exposed PBMCs in the placebo group, and that n-3 fatty acid supplementation prevented this reduction. There was a positive correlation between altered levels of plasma transthyretin, and the SPMs released from the PBMCs. Finally, in **Paper IV**, we investigated the therapeutic potential of a resolution-stimulating strategy. Using LC-MS-MS technique, we were able to study a broad range of lipid mediators, and found that MaR1 is also reduced in the entorhinal cortex of AD patients. MaR1, as well as LXA₄, RvD1, and NPD1 showed direct neuroprotective effects against staurosporine-induced cell death *in vitro*, and MaR1 specifically exerted phenotype-modulation effects on human microglial cells, and promoted the phagocytosis of A β .

In conclusion, we have demonstrated that the resolution pathway is altered in AD, and has a relationship with disease development and pathology. Novel therapeutic strategies based on stimulating resolution should be further investigated.

LIST OF SCIENTIFIC PAPERS

- I. **Xiuzhe Wang**, MingqinZhu, Erik Hjorth, Veronica Cortés-Toro, Helga Eyjolfsdottir, Caroline Graff, Inger Nennesmo, Jan Palmblad, Maria Eriksdotter, Kumar Sambamurti, Jonathan M. Fitzgerald, Charles N. Serhan, Ann-Charlotte Granholm & Marianne Schultzberg
Resolution of inflammation is altered in Alzheimer's disease.
Alzheimer's & Dementia. doi: 10.1016/j.jalz.2013.12.024.
- II. **Xiuzhe Wang**, Elena Puerta, Angel Cedazo-Minguez, Erik Hjorth, Marianne Schultzberg
Insufficient resolution response in the hippocampus of a senescence-accelerated mouse model - SAMP8.
Journal of Molecular Neuroscience. doi: 10.1007/s12031-014-0346-z
- III. **Xiuzhe Wang**, Erik Hjorth, Inger Vedin, Maria Eriksdotter, Yvonne Freund-Levi, Lars-Olof Wahlund, Tommy Cederholm, Jan Palmblad, Marianne Schultzberg
Effects of n-3 fatty acid supplementation on the release of pro-resolving lipid mediators by blood mononuclear cells: the OmegAD study
Submitted manuscript.
- IV. Mingqin Zhu, **Xiuzhe Wang**, Erik Hjorth, Romain A. Colas, Lisa Schröder, Ann-Charlotte Granholm, Charles N. Serhan, Marianne Schultzberg.
Roles of pro-resolving lipid mediators in Alzheimer's disease, neuronal cell survival and A β ₄₂ phagocytosis by microglia
Manuscript

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AD	Alzheimer's disease
ALX/FPR2	lipoxin A ₄ receptor/formyl peptide receptor 2
ANCOVA	analysis of covariance
ApoE	apolipoprotein E
A β	amyloid β
A β PP	amyloid β precursor protein
CA	cornu ammonis region
ChemR23	chemerin receptor 23
COX	cyclooxygenase
CSF	cerebrospinal fluid
CYP450	cytochrome P450
DAB	diaminobenzidine
DHA	docosahexaenoic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
FA	fatty acid
GPR32	G-protein coupled receptor 32
GWAS	genome-wide association studies
HDHA	hydroxy docosahexaenoic acid
HETE	hydroxy eicosatetraenoic acid
HEPE	hydroxy eicosapentaenoic acid
HLA-DR	human leukocyte antigen-type DR
HpDHA	hydroperoxy docosahexaenoic acid
HpETE	hydroperoxy eicosatetraenoic acid
IL	interleukin
IFN- γ	interferon- γ
LC-MS-MS	liquid chromatography-tandem mass spectrometry
LOX	lipoxygenase

LT	leukotriene
LX	lipoxin
MaR	maresin
MCI	mild cognitive impairment
MHC-II	major histocompatibility complex Class II
MMSE	mini-mental state examination
mTORC	mammalian target of rapamycin complex
NFκB	nuclear factor-κ B
NPD	neuroprotectin
NSAID	non-steroidal anti-inflammatory drugs
p-tau	phosphorylated tau
p70S6K	ribosomal protein S6 kinase
PBMC	peripheral blood mononuclear cell
PET	positron emission tomography
PG	prostaglandin
PMI	postmortem interval
PPAR-γ	peroxisome proliferator-activated receptor-γ
PS	presenilin
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RvD	resolvin D
RvE	resolvin E
SAMP8	senescence-accelerated mouse prone 8
SAMR1	senescence-accelerated mouse resistant 1
SCI	subjective cognitive impairment
SPM	specialized pro-resolving mediator
TTR	transthyretin
TxB ₂	thromboxane B ₂

1 INTRODUCTION

The human brain is perhaps the most amazing art of nature. It gives us the abilities to recognize the world and the mind, to memorize the tears and the smiles, to reform the world and remould the thought. However, these abilities wither as the brain becomes dysfunctional.

1.1 ALZHEIMER'S DISEASE

1.1.1 General aspects

Alzheimer's disease (AD) is a neurodegenerative disorder that causes the most common type of dementia. The history of AD dates back to more than 100 years ago, when the German psychiatrist and pathologist Alois Alzheimer first described the pathologies of brain atrophy, senile plaques and neurofibrillary tangles in the brain of a demented woman in 1906 [1]. In recent years, the 'golden standard' for determination of AD based on these two hallmarks in the post-mortem brain examination became considered as just one of several diagnostic tools, which include new developing guidelines based on clinical symptoms, imaging and laboratory examinations in favour of clinical practice [2,3].

There are about 35 million people suffering from dementia, among which more than half may be of the AD type, and the number is expected to grow to 150 million. Epidemiologically, AD can be divided into two groups, the sporadic and familial, of which the latter is clearly associated with strong familial heredity. Sporadic AD accounts for the major proportion (about 95%) of AD cases, and the familial form thus only represents a small minority of the afflicted population [4].

The clinical symptoms of AD are characterized by progressive loss of memory and cognition, believed to be associated with neuronal damage in the brain. AD can lead to inability with working and everyday life. In the late stage, the AD patient may need to be hospitalized due to difficulties with basic daily living activities, such as eating and bathing. The lifespan after the diagnosis of AD is usually less than 10 years [5,6]. AD does not only results in suffering for the patient, but also becomes a serious burden for the patient's family and the social healthcare system.

1.1.2 The evidence of inflammation in AD - Observations

In the 1980s, about 80 years after Dr. Alzheimer's description of AD, the presence of inflammatory reactions in the postmortem brain of AD patients was discovered [7-10]. Immunoglobulins and complement factors, representing immune responses, were found to be associated with senile plaques in the AD brain [7]. Later on, microglia, the cells representing the innate immune system in the brain, were found to be more reactive in the AD brain [8,9], and levels of interleukin (IL) -1, a classical inflammatory cytokine, found to be elevated [10]. The presence of abnormal inflammation in the AD brain has since been confirmed by other studies [11-16].

Signs of an inflammatory process in AD were not found only in the brain, but also in body fluids. In the cerebrospinal fluid (CSF) of AD patients, higher levels of pro-inflammatory cytokines such as IL-6, IL-1 β , tumor necrosis factor- α (TNF- α), and acute phase protein α -1-antichymotrypsin levels were reported, although some reports of decreased or unchanged levels have also been published (reviewed in [17]). Similar findings have been reported for the levels in serum/plasma samples (reviewed in [17]).

Besides these laboratory examinations on clinical materials from AD patients, strong support of elevated inflammation is also available from the live examination by clinical imaging techniques. Using positron emission tomography (PET) technique, it was possible to detect increased activation of microglia cells in the brain of living AD patients [18-20]. Activated microglia were found to be more numerous also in the brain of mild cognitive impairment (MCI) patients [21], who are believed to be at high convert rate to AD. The levels of activated microglia detected by this method did not differ between MCI and AD patients [21], suggesting an early role of inflammation in the development of AD.

Evidence of an early role for inflammation in the development of AD is also provided by epidemiological studies. In 1989, the first report was published regarding a lower incidence of AD in patients with rheumatoid arthritis [22], which is an autoimmune disease mainly affecting the joints and other peripheral organs. Patients with rheumatoid arthritis are usually prescribed with anti-inflammatory drugs, and hence, in a report from 1990, McGeer and co-

workers suggested that the use of anti-inflammatory drugs in these patients may be a protective factor against development of AD [23]. Later on, large cohort epidemiological studies on the association between use of anti-inflammatory drugs and AD have been published. The Rotterdam study, a longitudinal study following more than 7000 subjects, reported a relative risk for AD of 0.38 in users of non-steroidal anti-inflammatory drugs (NSAIDs), compared to those not using NSAIDs [24]. In the follow-up study, it was reported that 24 months medication with NSAIDs, or longer, was associated with a relative risk for AD of 0.20, compared to 0.83 when using NSAIDs for more than 1 month but less than 24 months [25].

Evidence for the involvement of inflammation in AD was also obtained from the field of genetics. Genome-wide association studies (GWAS) have identified polymorphisms of genes related to immune responses, such as CR1/CD35 and CD33, with strong association with AD (reviewed in [26]). A recent report by Raj T et al (2014) also indicated that expression quantitative trait loci for risk gene variants of AD were over-represented in monocytes, a leukocyte from the myeloid compartment, also the origin of brain microglia, indicating a vital role of the innate immune system in genetic risk factors for AD [27].

1.1.3 The role of inflammation in AD - Ideas

With the observation of inflammation in AD, it is of importance to consider its role, especially from an aetiological perspective, and below follows hypotheses regarding possible mechanisms by which inflammation may play a critical role in AD pathogenesis.

1.1.3.1 Inflammation and amyloid- β

Certain gene mutations have been identified to result in heredity of AD. These mutations include the genes for amyloid- β precursor protein (A β PP), and presenilin (PS) 1 and 2 proteins [4]. As indicated above, familial AD (FAD) only accounts for a minor proportion of all AD cases, and the proportion of cases with these three mutations is even less, representing approximately 55-70% of known FAD cases [4]. Thus, the aetiology of the majority of AD cases, especially the sporadic type, is still largely unknown.

Almost all the mutations found on the three genes in FAD are related to the increased production of amyloid β ($A\beta$) [28], the principle component of senile plaques found in the postmortem AD brain. Based on the genetic findings from the FAD, the $A\beta$ cascade hypothesis was formulated, suggesting that abnormal $A\beta$ accumulation is the central cause of AD type dementia [29,30]. Much research into AD is either directly focusing on $A\beta$, or indirectly related to it. $A\beta$, a peptide of about 36-43 amino acids, is produced from the $A\beta$ PP-presenilin complex through β - and γ -secretase [31].

Inflammation has been proposed to be a critical intermediate for the disease development as part of the $A\beta$ cascade hypothesis [31]. As described in the early reports on inflammation in the AD brain, the pro-inflammatory proteins and reactive microglial cells are associated with the senile plaques [7-10]. A common belief is that these inflammatory activities represent a response to the neurodegeneration in the brain. Furthermore, based on several *in vitro* studies (reviewed in [32]), the neuroinflammation is considered as a result of stimulation by the overabundant $A\beta$ in the AD brain. Microglia, together with astrocytes, can be activated by $A\beta$ through various sensors, including receptors such as toll-like receptors (TLRs) [33,34], receptor for advanced glycation endproducts (RAGE) [35-37], and formyl peptide receptor 2 (FPR2) [36,38-41]. The activated microglia and astrocytes release pro-inflammatory factors such as $TNF-\alpha$, $IL-1\beta$ and reactive oxygen species (ROS), that are able to damage neurons [42-46] or blunt neurogenesis [47]. Furthermore, activated microglia may even destroy neurons directly through a phagocytosis-like process [48]. Inflammation may thus serve as a mediator for $A\beta$ toxicity in the brain through the mechanisms described above.

However, stimulation in the opposite direction must also be considered, *i.e.* that inflammation is a promoter of $A\beta$ production, and thereby contributing to the neurodegenerative process. Disregarding the primary cause of inflammation, it is clear that inflammatory signals can regulate the production of $A\beta$ [49]. For example, the pro-inflammatory cytokine $IL-1\beta$ alone can enhance $A\beta$ PP processing [50], and stimulation by interferon- γ (IFN- γ), $IL-1\beta$ or $TNF-\alpha$ increased production of $A\beta_{40}$ and $A\beta_{42}$ [51,52]. The abnormally high $A\beta$ levels can then further stimulate inflammation that mediates toxicity, or possibly exerts direct detrimental

effects to the neurons [53]. Thus, inflammation and A β could promote each other and form a vicious circle in the pathogenesis of AD [54].

1.1.3.2 Inflammation and aging

While the A β cascade hypothesis is being more and more questioned for various reasons, *e.g.* failures of clinical trials based on reducing A β [55], new hypotheses are being generated. Recently, Hunter S et al (2013) has proposed that AD, both familial and sporadic, could be due to senescence of the neurons in the brain, and that A β secretion is a response to senescence [56]. Indeed, aging is perhaps the most accepted risk factor for AD [57]. Most AD cases are diagnosed after the age of 65 [58]. The risk to develop AD doubles every 5 years after the age of 65, and after 85 years, the risk is more than 20 times higher than that at 65 of age [59].

Signs of inflammation are present in the progress of aging. Serum levels of pro-inflammatory cytokines, such as IL-6 and TNF- α , are elevated with age [60,61]. Increased production of pro-inflammatory cytokines by blood mononuclear cells was found upon aging [62], and glial cells in the brain become more activated towards a pro-inflammatory status [63,64]. Some researchers concluded that aging is a process pushing inflammation, and pushed by inflammation, a phenomenon they termed ‘inflamm-aging’ [65,66]. According to this theory, the human body is continuously exposed to different types of stressors, such as radiation, antigenic, oxidative and thermal stimuli, that all can stimulate the immune system (especially the macrophages) during the entire life span. In this way, the inflammation level is growing chronically, and may impair pro-homeostatic mechanisms such as DNA repair systems and free radical scavenging. The balance between pro-inflammatory stressors and anti-inflammatory rescuers is important in determining if the aging process is ‘successful’. For example, in healthy centenarians, the balance between pro-inflammatory factors, such as IL-1 and IL-6, and anti-inflammatory factors, such as IL-10, plays a key role in their successful aging process [67,68]. As such, to balance the inflammatory and anti-inflammatory events could be an important strategy to achieve successful aging. On the other hand, age-related diseases, such as AD, could be associated with a disturbed balance between the two aspects.

1.1.3.3 Inflammation and ApoE

Apolipoprotein E (ApoE) is a 34kDa lipoprotein abundant in both periphery and brain. There are 3 major isoforms of the APOE gene allele in human: E2, E3 and E4, of which E3 is the most common form (about 75%) and E2 is the least common (about 8%) [69]. APOE4, which appears in about 14% of the population, is associated with both familial and sporadic AD [70,71], and confers a dose-dependent increase in the risk for AD, and decreases the age of onset [72].

The first described function of ApoE was the transport of lipids, but further studies have shown it to be involved in many other functions apart from lipid transport, including immune modulation [73]. Interestingly, the brain appears to be the second major source of ApoE protein after the liver [74]. Immunohistochemistry shows that staining for ApoE in the AD brain is associated with the senile plaques, and that it is increased in reactive astrocytes [75,76], indicating a link between ApoE, A β and inflammation. AD patients carrying the APOE4 allele were found to have an increase in activated microglia in the brain [77]. Transgenic mice with human APOE4 displayed higher levels of pro-inflammatory responses to lipopolysaccharide (LPS), both in the brain and periphery, compared with the mice carrying APOE3 isoform [78], in line with the findings in human that young and middle-aged APOE4 carriers had higher plasma levels of pro-inflammatory cytokines, which may be associated with a risk for AD in late life [79]. In cellular studies, inflammatory reactions in microglia upon stimulation with secreted A β PP (sAPP- α) can be attenuated by the ApoE3 protein, but not by ApoE4 [80]. In a clinical study, the risk for AD was reduced by NSAIDs only in APOE4 carriers, and selective A β -lowering NSAIDs did not alter the risk-reducing effect compared to non-A β -lowering NSAIDs in these patients [81], indicating an effect of the anti-inflammatory treatment that was independent of A β .

1.1.3.4 Inflammation and tau

In the debate about key players in AD pathogenesis, the neurofibrillary tangles consisting of hyperphosphorylated tau, cannot be forgotten, as it represents one of the two pathological hallmarks. While the normal physiological function of A β has not been well understood [82],

tau is known to act as a microtubule-binding protein that is essential for maintaining the stability of the neuronal cytoskeleton, and for normal cellular traffic [83]. Instead of binding to microtubules in a normal condition, highly phosphorylated tau (p-tau) that accumulates in a neuron forms paired helical filaments that are further turned into neurofibrillary tangles, disturbing the normal neuronal shape and function. While the A β cascade hypothesis is being challenged, interest in the importance of tau in for AD pathogenesis has been growing. As p-tau levels seem independent of A β [84-86], and appear to change earlier than A β [87,88], researchers have proposed that drugs targeting tau would be preferential as disease-modifying drugs, while A β -targeting drugs may be more suitable for disease prevention [89].

Also in connection to tau pathology, inflammation is a big part of the blue print. Similarly to A β , tau pathology can induce inflammation in the brain [90-92]. On the other hand, inflammation modulates tau. In the gene-manipulated mice that express human mutant tau genes, activation of microglia or chronic expression of pro-inflammatory cytokines, such as IL-1 β , resulted in exaggerated p-tau pathology [93-95]. Interestingly, an immunosuppressant drug that can reduce microglial activation decreased the p-tau abnormalities [94]. Increased levels of total tau also represent a feature of the AD brain [96]. In vitro studies showed that pro-inflammatory cytokines, or conditioned medium from activated microglia, increased tau production, and this could be attenuated by anti-inflammatory drugs or IL-10 [97]. At the molecular level, tau homeostasis is based on the balance between phosphorylating and de-phosphorylating activities. Studies on postmortem AD brains showed that tau phosphorylating kinases such as cyclin-dependent kinase 5 (cdk5) and p38/mitogen-activated protein kinase (p38/MAPK) are increased, and that tau de-phosphorylating phosphatases such as protein phosphatase 2A (PP2A), are reduced [98]. Inflammatory signals are known to enhance p-tau levels through activating the phosphorylation kinases [86,99].

1.1.4 Inflammation in the intervention of AD - Practices

Based on the above findings, interventions of AD through an inflammation-focused strategy have been tested. The use of NSAIDs is the most relevant example. Inspired by the epidemiological findings, various types of NSAIDs have been tested for prevention or treatment effects in AD. In animal models of AD, NSAIDs have been shown to exert some

beneficial effects by reducing AD-related pathologies, and improving cognitive functions [100,101], but contrasting results that NSAIDs worsen AD-related pathologies have also been reported ([102,103] and reviewed in [104]). In clinical trials with NSAIDs on AD-patients, the results have been inconsistent, and almost no preventive effect, or reduction of AD symptoms could be concluded [105]. In contrast, the treatment with some NSAIDs could even increase the risk of AD [106,107], or worsen AD severity [108]. Moreover, many of the clinical trials with NSAIDs had to be terminated due to severe side effects, such as bleeding and cardiovascular diseases. The complicated results from clinical trials and epidemiological studies require further investigation of the mechanisms for NSAIDs' activities in humans with different disease conditions.

Another type of treatment strategy related to inflammation is the use of polyunsaturated fatty acids (PUFAs), especially the n-3 fatty acids (FAs). It is well accepted that intake of n-3 fatty acids, such as docosahexaenoic acid (DHA, 22C:6(n-3)) and eicosapentaenoic acid (EPA, 20C:5(n-3)), benefits cardiovascular and other healthy aspects, many of which are attributed to anti-inflammatory effects [109]. Again, in animal studies, n-3 FA supplementation significantly reduced AD-related pathologies such as A β and p-tau [110], and improved learning and memory [111]. However, it becomes more complicated in clinical setting. The first large randomized, double-blind, placebo-controlled clinical trial was the OmegAD study [112]. In this study, 174 AD patients, of the 204 enrolled, concluded the study. The patients were randomized to orally take placebo or a supplement of 1.7 g DHA and 0.6 g EPA daily for the first 6 months, and in the following 6 months all patients were given this n-3 FA supplement. The overall conclusion from the OmegAD study is that there was no improvement in cognitive function in patients that received n-3 FA supplement. However, when analysing a sub-group of patients with MMSE > 27 at baseline, n-3 FA supplementation significantly reduced the MMSE decline rate. Within the same year, a study on supplementation with arachidonic acid (AA) and DHA showed improvement in cognitive function in MCI, but not in AD patients [113]. Similarly, a multi-centre study in which 225 drug-naïve AD patients from five different countries were treated with placebo or a supplement with DHA (1.2 g) and EPA (0.3 g) daily for about 3 months, showed no

improvement in cognitive function, but in patients with MMSE of 24-26 at baseline, the n-3 FA supplement improved a specific cognitive function, the delayed verbal recall [114]. The authors performed another multi-centre study, in which the inclusion criterion was MMSE \geq 20, and confirmed that the same supplementation could improve cognitive function [115]. Although the studies could not provide a significant effect in all AD patients, they showed some indication as to the potential for PUFA-based anti-inflammatory strategy against early stages of dementia. The complexity of the results stimulated further basic and clinical research, presented in the following sections.

1.2 RESOLUTION OF INFLAMMATION

1.2.1 Pro-resolving lipid mediators and the new concepts

While NSAIDs are extrinsic tools made by pharmaceutical companies, resolution of inflammation, is the intrinsic system designed by nature with which inflammation is controlled and terminated. Acute inflammation is usually a defence reaction of the body to fight invading stimuli, such as pathogens and chemicals, or injury. In this sense, inflammation is a protective process. However, inflammation needs to be resolved, as the mechanisms by which harmful stimuli are destroyed and neutralized, are also be harmful to the tissue. Resolution of inflammation is not a novel concept. In general, during resolution of inflammation, 1) recruitment of inflammatory cells into the inflammation site is stopped, and/or the cells are switched to an anti-inflammatory phenotype, 2) non-phlogistic phagocytosis of stimuli and cell debris by macrophages is promoted, 3) pro-inflammatory signals are ceased and the pro-inflammatory mediator levels are down-regulated, 4) anti-inflammatory signals and local cell/tissue survival signals are up-regulated, and 5) repair/regeneration of inflamed tissue is initiated [116]. Through such a process, the inflamed tissue/organ returns to homeostasis, and regains normal function.

It was believed that the resolution of inflammation is a passive process following the removal or disappearance of inflammatory stimuli [117]. While some of the events in the resolution stage could be explained by the withdrawal of harmful stimuli, many questions at the cellular and molecular level remained unclear [117]. For example, in acute inflammation, it was

known that recruitment of macrophages into the inflammation site occurs a few hours later than the recruitment of neutrophils, but the initial mechanism of this sequential recruitment of inflammatory cells was not clear [117]. Questions regarding the intrinsic mechanism for pushing infiltrated granulocytes into apoptosis or to lymph nodes, and for the shift in macrophages to different phenotype spectra, were not clarified [117].

Not until recent years has important information been provided on the mechanism of resolution, largely due to the serial discovery of specialized pro-resolving lipid mediators (SPMs) [118]. The SPMs are small molecular weight lipids derived from PUFAs, including AA, DHA and EPA. Upon the secretion of these mediators into the inflamed tissue, resolution of inflammation is initiated and promotes the return of tissue homeostasis [118].

1.2.1.1 Lipoxins and programmed resolution

Lipoxins (LXs) are the first identified SPMs. The discovery of LXs was in the early 1980s in the lab of Nobel Laureate, Professor Bengt Samuelsson, who discovered the AA metabolites prostaglandins (PGs) [119,120]. Similarly to PGs, LXs are also metabolites from AA. But unlike PG biosynthesis that requires cyclooxygenases (COXs) to catalyze AA, LXs were found to alternatively use lipoxygenases (LOXs) [120].

There are two major types of LXs, LXA₄ and LXB₄. The name ‘lipoxin’ is based on the key role of ‘lipoxxygenases’ involved in their biosynthesis [119] (Figure 1). There are two major biological pathways for the synthesis of LXs. One of them involves the 15-LOXs (including 15-LOX-1 and 2), that first oxygenize AA into 15S-hydroperoxy eicosatetraenoic acid (15S-HpETE), and 5-LOX for conversion of 15S-HpETE to 5S,15S-diHpETE, which is finally converted into LXs (Figure 1). The other pathway is initiated by 5-LOX to catalyze AA to LTA₄, which will be further converted by 12/15-LOX into LXs (Figure 1). Notably, the 5-LOX initiated AA pathway is also able to produce leukotrienes. The epimer of LXA₄, 15-epi-LXA₄, was found to be synthesized from AA through the aspirin-triggered (AT) COX-2 pathway (Figure 1) [121].

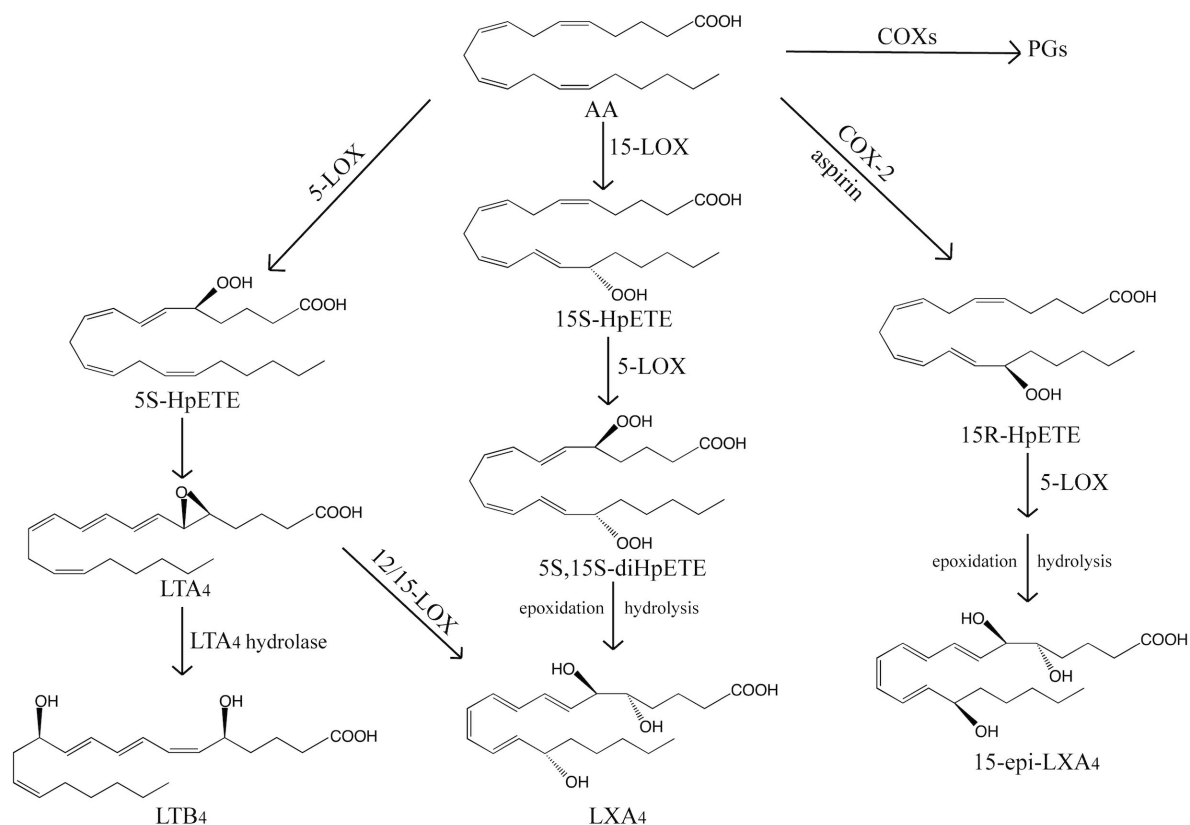


Figure 1. Biosynthesis of lipoxins and other lipid mediators from AA.

The biological functions of LXs, especially LXA₄, were intensively studied soon after their discovery. LXA₄ is perhaps the best studied SPM not only because of the early time of its discovery, but also truly for its biological significance. The first evidence of their pro-resolving effects came from a leukotriene B₄ (LTB₄) induced skin inflammation model in the hamster cheek [122]. In this model, topically applied LXA₄ dramatically reduced leukocyte infiltration and plasma leakage in the skin air pouch infused with LTB₄ or PGE₂. As the authors predicted, the pro-resolving role of LXs were robustly demonstrated afterwards. In various models *in vivo* and *in vitro*, LXA₄ has been able to: cease inflammatory cell infiltration, attenuate inflammation-induced tissue damage, down-regulate pro-inflammatory cytokines and oxidative stressors (ROS), and promote phagocytosis and anti-inflammatory signals, such as IL-10 (reviewed in [116]). LXA₄ and its epimer exert their biological actions through a G-protein coupled receptor, formyl peptide receptor 2 (ALX/FPR2) [123], for which also Aβ is a ligand.

A milestone finding for both LXA₄ itself, and the idea of a programmed and active resolution process was established in 2001 [124]. In a model of acute skin inflammation induced by air pouch in mice, LTB₄ appeared to be the first peak of increased pro-inflammatory mediators in the exudate, followed by PGE₂. About 1-2 hours after the peak of these pro-inflammatory mediators, the levels of LXA₄ were increased to a peak about 30-200 times higher than peaks of its AA siblings, LTB₄ and PGE₂. At the time of the LXA₄ peak, the levels of LTB₄, PGE₂ and neutrophil numbers were lower than their maximum levels, which were still much higher than baseline. This ‘class-switching’ of pro-inflammatory mediators to pro-resolving LXA₄ during an on-going inflammatory response strongly supported that resolution of inflammation is an active and intrinsic system that controls inflammatory response, and is programmed from the beginning of an inflammatory response (Figure 2) [125].

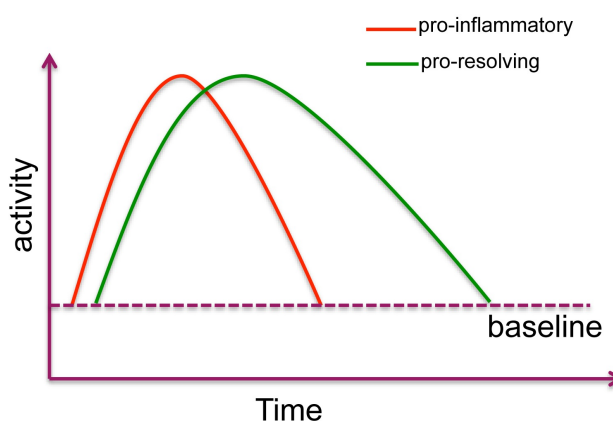


Figure 2. Programmed resolution in inflammation

1.2.1.2 Resolvins

Inspired by the findings from LXs as pro-resolving metabolites from a PUFA, researchers identified resolvin (Rv) series from n-3 FAs, DHA and EPA, in the early 2000s, and named them according to their potent pro-resolving functions. The two series are RvDs, of which the precursor are from DHA, and RvEs that are derived from EPA [126].

Resolvin D series

In the biosynthesis of RvD1, 15-LOX-1 catalyses the conversion of DHA to 17S-HpDHA, which is oxygenized by 5-LOX and converted to RvD1 by further epoxidation and hydrolysis (Figure 3) [127]. Similar to LXs, there are also RvD epimers (AT-RvDs) derived in aspirin-triggered COX-2 pathway (Figure 3) [127].

There are six types of RvDs discovered in the human, i.e. RvD1-RvD6 [128], of which RvD1 is the most studied so far. Similarly to LXA₄, RvD1 exerts potent pro-resolving functions,

such as arresting granulocyte infiltration, down-regulating ROS and pro-inflammatory cytokines, and enhancing phagocytosis and expression of anti-inflammatory cytokines (reviewed in [116]). Of importance, RvD1 can modulate the expression of microRNAs (miRNAs), which play a vital role in controlling many aspects of cellular processes [129]. Moreover, RvD1 is able to modulate class-switching of the AA cascade, *i.e.* by inhibition of LTB₄ production and enhancing LXA₄ synthesis, through limiting the nuclear localization of 5-LOX [130]. This provided the evidence that there may be a network regulation between SPMs. RvD1 was found to provide its biological functions through G-protein coupled receptor 32 (GPR32), as well as ALX/FPR2 [131], also serving as receptor for LXA₄. Although LXA₄ is also able to bind to GPR32, no specific functional outcome of the binding has been confirmed [131].

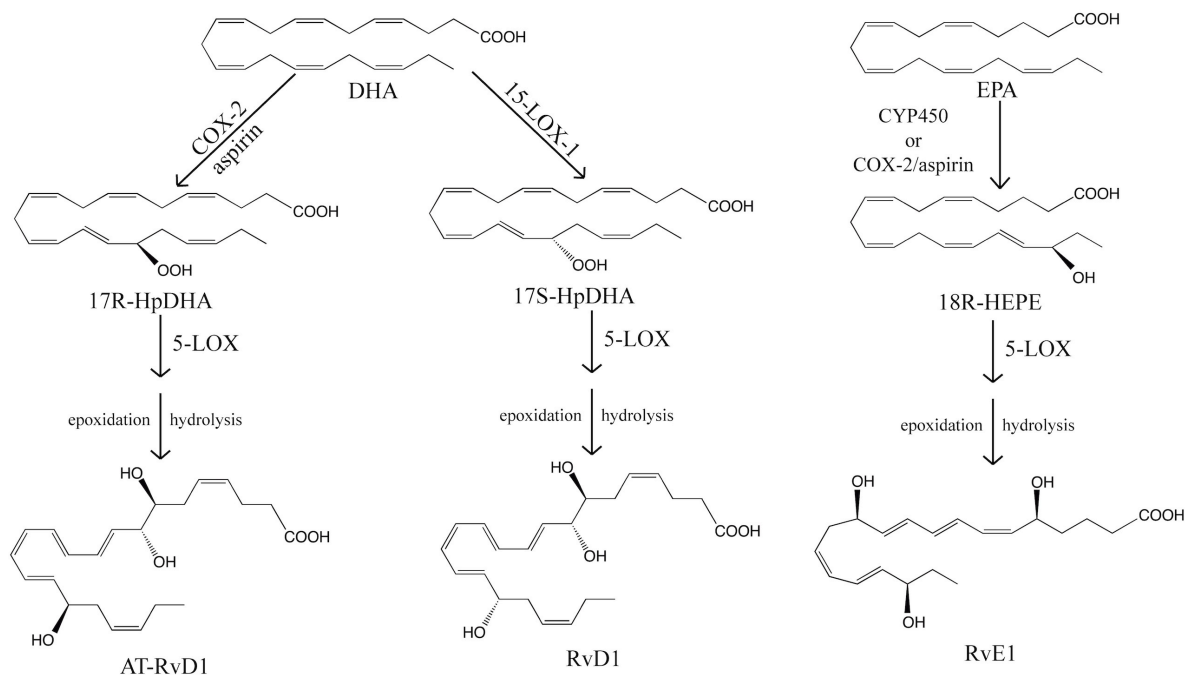


Figure 3. Biosynthesis of resolvins from DHA and EPA.

Resolvin E series

The synthesis of RvE1 can be initiated by either cytochrome P450 (CYP450), or aspirin-modified COX-2 enzymes, converting EPA into 18R-HEPE, which is further metabolized by 5-LOX (Figure 3) [132,133].

Three RvEs have been discovered, *i.e.* RvE1, RvE2 and RvE3 [128], of which RvE1 has been well characterized. RvE1 has been found to reduce dendritic cell migration and their

production of IL-12, inhibit nuclear factor- κ B (NF κ B), and promote granulocyte apoptosis and clearance (reviewed in [116]). Similarly to RvD1, RvE1 is able to increase the production of LXA₄ [134]. The G-protein coupled receptor chemerin receptor 23 (ChemR23) was found to mediate RvE1 induced activities [135]. Moreover, RvE1 is also able to bind to, and acts as an antagonist of, the LTB₄ receptor 1 (BLT1), which mediates pro-inflammatory LTB₄ functions [136].

1.2.1.3 Neuroprotectins

Neuroprotectins (NPDs, equal to the name protectins) are the SPMs first discovered in the nervous system, providing neuronal protection. Therefore, the name ‘neuroprotect’ was chosen, with ‘in’ for its inhibition of pro-apoptotic signals, and ‘D’ for being derived from DHA [137,138]. The brain and retina have very high levels of DHA that is essential for their development, maintenance and function, and the idea was formulated that specific functional derivatives from DHA may exist in the nervous system. In the mouse brain, and in a retinal pigment epithelium (RPE) cell line, NPD1 was discovered using liquid chromatography-tandem mass spectrometry (LC-MS-MS) technique [137,138].

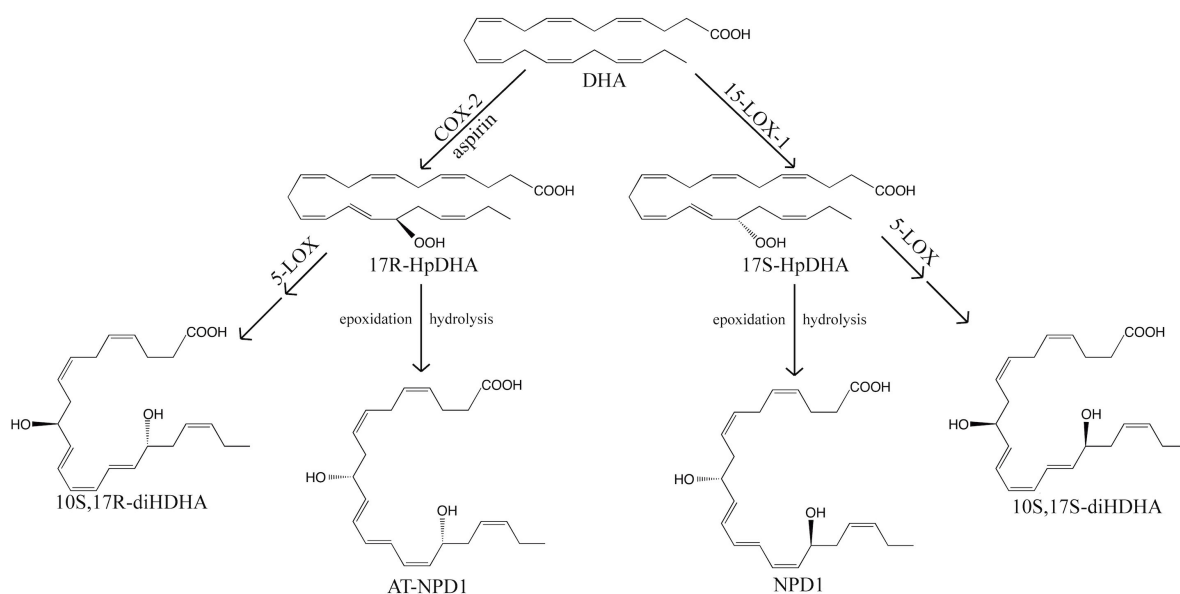


Figure 4. Biosynthesis of neuroprotectins from DHA.

In the synthetic pathway of NPD1, 15-LOX-1 converts DHA to 17S-HpDHA, which is converted to NPD1 by further epoxidation and hydrolysis (Figure 4) [137,138]. Notably, 5-LOX can also participate in this pathway, and convert 17S-HpDHA into 10S, 17S-diHDHA

(PDX), which are much less efficient than NPD1 (Figure 4) [137,139]. There is also an aspirin-triggered epimer for NPD1, AT-NPD1 (Figure 4) [140].

NPD1 was early discovered to exert potent neuroprotective activities, such as in the mouse stroke-reperfusion model, by reduction of brain ischemia volume, and infiltration of granulocytes to the brain, and it was also shown to inhibit NF κ B activation and COX-2 expression [137]. In cultures of RPE cells, NPD1 was shown to protect from apoptosis induced by oxidative stress, through up-regulation of anti-apoptotic protein Bcl-2, and attenuated caspase-3 activation [138]. NPD1 has also been shown to inhibit COX-2 expression and NF κ B activation in IL-1 β stimulated neuronal cell models [137,138].

1.2.1.4 Maresins

Maresins (MaRs) are the SPMs found most recently, i.e. in 2009 [141]. They are derivatives from DHA, and were named maresins due to the original discovery in macrophages.

Synthesis of MaR1 includes oxygenation of DHA by 12-LOX into 14S-HpDHA, followed by epoxidation and hydrolysis (Figure 5) [141]. 5-LOX can alternatively convert 14S-HpDHA to 7S, 14S-diHDHA (Figure 5), which possesses some, but weaker pro-resolving function than MaR1 [139,141].

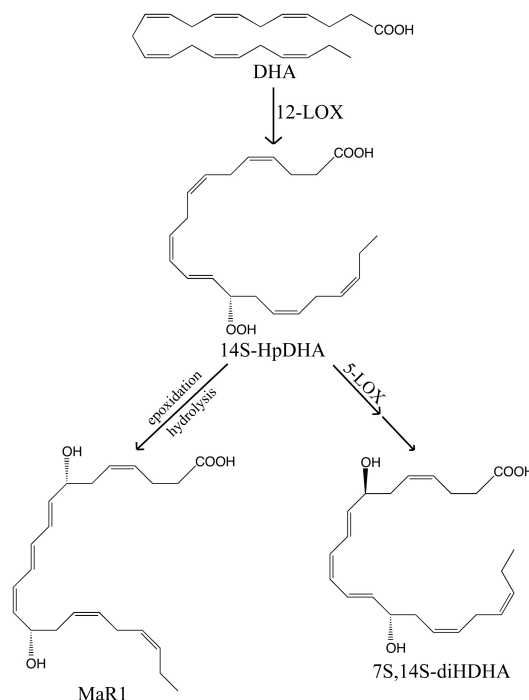


Figure 5. Biosynthesis of MaR1 from DHA

MaR1 was identified first, and MaR2 was discovered this year [142]. MaR1 is able to reduce granulocyte infiltration to an extent comparable to NPD1 and RvE1 [141]. As a product from macrophages, MaR1 shifts macrophages from a pro-inflammatory phenotype (M1) to an anti-inflammatory/phagocytic phenotype (M2), and inhibits LTA₄ hydrolase [143], which is involved in the production of LTB₄ from AA. Interestingly, MaR1, together with RvE1, was

found to promote tissue regeneration in surgically injured worms [144], providing evidence of pro-regenerative function of SPMs.

1.2.2 Resolution of inflammation in health and diseases

1.2.2.1 Roles of SPMs in normal physiology

As SPMs were discovered in animal models and cellular studies, it is of importance to confirm their presence in humans. It has been established that almost all the SPMs could be identified in healthy human serum [128], confirming the significance of SPMs in human health.

Milk, the first food for a human being, contains high levels of SPMs (in ng/ml) in the first month of lactation [145]. LXA₄ (~ 15-22 ng/ml) appears to be the most prevalent among the SPMs found in milk, followed by RvD1 and RvE1. Moreover, the levels of these SPMs remained stable, regardless of the decreasing levels of their precursors AA and DHA. These findings indicate the potential importance of SPMs in the development of infants.

The significance of SPMs in adults is also established. Physical exercise, especially resistance training, can induce acute inflammation that regulates muscle growth and maintenance. In this acute inflammation model, researchers have demonstrated the programmed resolution for the first time in humans [146]. In this study, serum samples were collected from healthy adults at 0-3 and 24 h after resistance exercises. Inflammatory mediators, such as thromboxane B₂ (TxB₂), LTB₄ and PGE₂, were increased in the acute phase, and accompanied by an enhanced production of SPMs.

Thus, it is clear that SPMs are involved in the normal physiology of humans, and the roles of SPMs may be of importance in inflammation-related diseases.

1.2.2.2 Roles of SPMs in peripheral diseases

Asthma is an allergic disease of the airways, associated with chronic inflammation. We have recently shown that airway allergy can increase the phosphorylation of tau protein, and the expression of inflammatory genes, in the brain [147,148]. In patients with severe asthma, the levels of LXA₄ [149,150] and NDP1 [151,152] are reduced. Administration of LXA₄, RvD1,

RvE1 or NPD1 in animal models of asthma, reduced airway hyper-responsiveness, decreased pro-inflammatory mediator levels, and inhibited the infiltration of eosinophils into the lungs [134,151,153-155].

Arthritis, such as osteoarthritis and rheumatoid arthritis, are chronic inflammatory diseases that affect the joints. As indicated above, long-term use of NSAIDs in patients with rheumatoid arthritis was associated with reduced prevalence of AD [23-25,156]. Treatment with SPMs in animal models of arthritis, including LXA₄ [157] and RvD1 [158], could significantly relieve the symptoms of joint degeneration, probably *via* decreasing levels of TNF- α , IL-1 β and LTB₄.

Diabetes mellitus (diabetes in short) is a metabolic disease that is caused by disturbance of insulin production or signalling, and obesity is a great risk factor for type 2 diabetes, the insulin resistant type [159]. Both diabetes and obesity are associated with inflammation, which serves as the link between the two diseases [160,161]. Notably, inflammation and insulin resistance are both associated with diabetes and AD, and type 2 diabetes doubles the risk to develop AD [162]. Pioglitazone, a drug that is commonly used to enhance insulin sensitivity in type 2 diabetes, was shown to increase plasma levels of LXA₄ epimer in diabetic patients, indicating a potential link between LXA₄ and the treatment effects of pioglitazone [163]. In addition, LXA₄ treatment reduced age-related increase of IL-6, enhanced IL-10 production, as well as increased insulin sensitivity-related genes [164]. Furthermore, RvE1 was able to reduce LPS-induced inflammation, and apoptosis in human pancreatic islets induced by pro-inflammatory cytokines [165]. In diabetic wounds, the levels of NPD1 and RvD1 precursors were reduced, and treatment with NPD1 or RvD1 promoted wound healing [166,167]. In addition, RvD1 could increase insulin sensitivity in obese-diabetic mice [168].

The emerging roles of SPMs and resolution in these AD-related peripheral diseases indicated potential links between resolution and AD.

1.2.2.3 Roles of SPMs in diseases of the nervous system

Pain is a symptom that can be caused by many diseases, but it is always sensed through the nervous system in humans. The incidence of both pain and AD is increased with age, and pain may be undertreated in AD patients, due to inability to report pain [169]. LXA₄, RvD1, RvE1, NPD1 and MaR1 were each shown to attenuate pain signalling through central and peripheral pathways in various inflammatory or traumatic pain models [144,170-173].

Stroke, known as a cause of dementia, is a disease of the brain due to ischemic or haemorrhagic disturbance of the brain blood flow. Biosynthesis of NPD1 was found to be increased 1 h after ischemic stroke, and administration of NPD1 or AT-NPD1 reduced the brain infarct volume, improved neurological function, and promoted long-term repair mechanisms [137,140,174,175]. Similarly, treatment with LXA₄, or its analogue, was also able to decrease the infarct volume and inflammatory mediators, ameliorate blood-brain barrier dysfunction and ischemia/reperfusion injury, and to enhance neurological recovery [176-181].

1.3 RESOLUTION OF INFLAMMATION AND AD

In AD, the proportions of AA and DHA in total FAs are decreased in different brain regions [182]. However, the consequential effects of these changes have not been clarified. As introduced above, a disturbance of the SPM production due to the decreased levels of their precursors is a reasonable result. The first light of resolution in AD was shed by NPD1 studies in 2005. Lukiw *et al.* reported that in the cornu ammonis region 1 (CA1) of the hippocampus, the levels of NPD1 and DHA were significantly reduced in AD compared to age-matched controls [52]. The authors also showed that NPD1 provided neuroprotection against A β ₄₂ induced cytotoxicity in neuron-glia co-cultures, through an up-regulation of anti-apoptotic gene expression and down-regulation of pro-apoptotic gene expression [52]. Later on, Medeiros *et al.* demonstrated that AT-LXA₄ treatment reduced AD-like pathologies, improved synaptic density and modulated glial cell functions, in AD transgenic mice [183,184]. Thus, considering the inflammation in AD, and the roles of SPMs in resolution of inflammation, we believe that a further insight into the resolution pathway in AD is needed to understand its possible role therein.

2 AIMS

The main aim of this thesis is to provide an overall view of resolution of inflammation in AD, from the aspects of observations, ideas, practices and future ideas.

The specific aims are:

Paper I: To characterize the status of resolution in the hippocampus and CSF of AD patients, and the possible relation to the stage of AD.

Paper II: To investigate how resolution is changed upon normal aging, and upon abnormal aging with AD-like symptoms and pathologies, using an animal model.

Paper III: To investigate if an anti-inflammatory treatment strategy, i.e. supplementation with n-3 fatty acids can influence the resolution of inflammation in AD patients, and how the influence may be related to other treatment effects.

Paper IV: To further characterize the profile of SPMs in entorhinal cortex of AD patients, and explore whether stimulation with SPMs may have functional effects in neuronal and glial cell models, in order to provide evidence for future development of therapeutic strategies based on SPMs.

3 MATERIALS AND METHODS

3.1 HUMAN, ANIMAL AND CELL MODELS

3.1.1 Human postmortem brain samples

The central pathological changes of AD are in the brain, and thus the AD brain is of the most interest when investigating a new factor/pathway in AD. Obtaining biopsies from a human brain is of obvious difficulty, and thus autopsy (postmortem) brain tissue becomes a valuable source. The age at death and the postmortem interval (PMI) are two important factors that may influence the analysis of postmortem samples, and need to be matched between the experimental groups. Postmortem human brain samples in **Paper I and IV** were obtained from the Brain Bank at Karolinska Institutet. In **Paper I**, hippocampal samples from 10 AD patients and 10 non-AD subjects without significant neurological disorders (control subjects) were used. The age at death and the postmortem interval (PMI) did not differ between the AD and non-AD group. In **Paper IV**, samples of entorhinal cortex from 7 AD patients and 7 control subjects were analysed. No difference was found regarding age and PMI (PMI from one subject was not available) between two groups. Severity of pathology is another factor to be considered when collecting postmortem samples. In both studies, all the AD brains were evaluated as Braak stage V-VI (except one case was stage III-IV), and there were no significant pathological changes in the control brains.

3.1.2 Human CSF samples

CSF is the body fluid secreted by the choroid plexus. It covers the entire brain and spinal cord, and actively interacts with interstitial fluid of the brain parenchyma. Because changes of CSF components can reflect the changes in the brain and can be ‘biopsied’ through lumbar puncture, CSF is a commonly used sample for neurological examinations. In **Paper I**, CSF samples from 15 AD patients, 20 MCI patients, and 21 individuals with subjective cognitive impairment (SCI), were obtained from the Memory Unit of Geriatric Clinic at Karolinska University Hospital, Huddinge. The age of the AD group is 68 ± 10 years, and that of MCI group is 66 ± 10 years (mean \pm SD). The age of the SCI group (57 ± 5 years) was younger

than the AD and MCI group. All of these 56 patients underwent routine clinical examinations including MMSE test and measurement of CSF p-tau (threonine 181).

3.1.3 The OmegAD study and blood mononuclear cells

Paper III is a second outcome sub-study from the OmegAD study, which is a double-blind, placebo-controlled, randomized clinical trial. The trial enrolled 204 AD patients from the beginning, and 174 patients completed the trial. Patients were randomized into two groups for the first 6 months, the n-3 FA supplement group receiving encapsulated 1.7 g DHA and 0.6 g EPA (EPAX1050TG; Pronova Biocare A/S, Lysaker, Norway) daily, and the placebo group receiving 1 g corn oil capsules (containing 0.6 g linoleic acids) daily. Vitamin E, an antioxidant, was also equally supplemented with the capsules to all the patients. After 6 months, all the patients received the n-3 FA supplementation for another 6 months. The primary outcomes have been reported previously [112]. A sub-population of 17 patients was included in **Paper III**. Two of the patients dropped out during the first 6 months trial, and finally 15 patients concluded the study, of which 8 patients (3 females), aged 72.5 ± 8.2 years (mean \pm SD) received the n-3 FA supplementation, and 7 patients (2 females), aged 70.4 ± 6.6 years (mean \pm SD) received placebo. There was no difference in age, MMSE score, plasma DHA and EPA levels, blood pressure, body weight or intake of aspirin, between the groups. Blood samples were taken before and after the first 6 months trial.

Blood mononuclear cells, including monocytes, T-lymphocytes, B-lymphocytes and natural killer (NK) cells, represent the immune system in the peripheral blood. While not possible to directly analyse brain cells from living patients, PBMCs were chosen as an *ex vivo* model to investigate the effects of the clinical trial on the inflammatory response to a stimulus. Moreover, PBMCs have been found to participate in the AD-related changes in the brain [185-190]. The venous blood samples obtained from the patients were treated with EDTA to prevent coagulation, and the PBMCs containing about 15% monocytes and 85% lymphocytes were isolated by gradient centrifugation (Nycomed Pharma, Oslo, Norway).

3.1.4 SAMP8 and SAMR1 mice

The senescence-accelerated mouse prone 8 (SAMP8) is a non-transgenic mouse strain with an abnormal aging progress, which displays AD-like pathologies and symptoms (reviewed in [191]). The senescence-accelerated mouse resistant 1 (SAMR1) is a mouse strain with a relatively normal aging progress, which has no AD-like changes. SAMP8 and SAMR1 mice were generated by phenotype difference from the AKR/J mouse strain [192,193]. SAMP8 is mainly considered as a model for aging, because of the accelerated aging process, and the multiple organ abnormalities [194]. However, it is also widely used as an AD-related model, as the SAMP8 mice display AD-like pathologies and symptoms, such as increased A β and p-tau, neuronal loss, and impaired spatial memory [195].

In **Paper II**, male SAMP8 and SAMR1 mice of 2 and 9 months age were used for the studies. For biochemical assays, the mice were injected intraperitoneally (i.p.) with a lethal dose of pentobarbital sodium. The brain was immediately removed, and the hippocampus was dissected out bilaterally and kept at -80°C until further processing. For morphological analysis, the mice were anesthetized with pentobarbital sodium i.p., and perfused with 4% paraformaldehyde via the left ventricle. The perfused brains were then kept in 4% paraformaldehyde at 4°C overnight, and transferred to 10% sucrose until sectioned.

3.1.5 SH-SY5Y neuroblastoma cell line

SH-SY5Y (**Paper IV**) is a human neuronal cell line derived from bone marrow autopsy of an adrenergic neuroblastoma patient. The undifferentiated SH-SY5Y cells are neuroblast-like and highly proliferative, and can be differentiated by various agents. Sequential culture with retinoic acid (RA) and brain derived neurotrophic factor (BDNF) differentiates SH-SY5Y cells into a cholinergic neuron-like cell population, which also possesses some dopaminergic properties [196]. The cells were cultured in DMEM/F12 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C in humidified air containing 5% CO₂. The cultured cells were differentiated with 10 μ M RA for 5 days. The differentiated cells were harvested with enzyme-free dissociation buffer, and seeded at 20 000 cells/well in a 48-well plate coated with Matrigel. The cells were allowed to attach to the plate for one day, and then

the culture medium was replaced with serum-free medium containing 25 ng/ml BDNF. Experiments were performed 5 days thereafter.

3.1.6 CHME3 microglia cell line

CHME3 is a human microglial cell line derived from human embryonic tissue via transfection with the SV40 large T antigen, which makes the microglia immortal and proliferate spontaneously [197]. In **Paper IV**, CHME3 was obtained as a kind gift from Professor Marc Tardieu (Université Paris South). The cells were cultured in DMEM/high glucose medium supplemented with GlutaMaxII (Life Technologies, Stockholm, Sweden) and 10% FCS. The culture medium was changed twice per week. The cells were split at 80% confluence, and seeded into 6-well plates for experiments and propagation.

3.2 EXPERIMENTS EX VIVO AND IN VITRO

3.2.1 Release of SPMs from PBMCs ex vivo

In **Paper III**, the cultured PBMCs were incubated with A β ₄₀. A β ₄₀ is a more abundant form in plasma than other A β peptides [198], and it has been shown that A β ₄₀ can reduce the production of IL-10 by PBMCs [199]. A β ₄₀ peptide (Bachem, Heidelberg, Germany) was dissolved in dimethyl sulfoxide (DMSO), and added to the cultures at a final concentration of 7 μ M, as this concentration of A β ₄₀ was shown to be more absorbed by cellular lipid membrane [200], without inducing significant cell death *in vitro* [201]. A β ₄₀ has been shown to increase lipid peroxidation [202], which may be linked to SPM biosynthesis. DMSO (1%) was used as vehicle. After 22 h incubation at 37°C with 5% CO₂, the cell cultures were centrifuged, and the supernatants were collected for further analysis of lipid mediators.

3.2.2 Effects of SPMs on the survival of SH-SY5Y cells

In **Paper IV**, to analyse the effect of SPMs on neuronal survival, the differentiated SH-SY5Y cells were treated with 100 nM staurosporine (STS), a protein kinase inhibitor widely used for inducing neuronal apoptosis [203]. The SPMs MaR1, LXA₄, RvD1 and NPD1, at a range of 0 - 0.5 μ M, were added to the cultures immediately prior to addition of STS. The treatment

with SPMs was repeated by addition to the cultures at 6 and 24 h, and viability and cell death were analysed at 48 h. The effects of SPMs alone in the absence of STS were also analysed.

3.2.3 Effects of SPMs on phagocytosis of A β ₄₂ by CHME3 cells

In **Paper IV**, to assess the effects of SPMs on phagocytosis of A β ₄₂, CHME3 cells (100,000 cells/well) were seeded into 6-well plates, and incubated with 1 μ g/ml HiLight488-conjugated A β ₄₂ (Anaspec, Fremont, USA), together with a range of concentrations (0 - 100 nM) of SPMs for 1 and 6 h. Phagocytosis of A β ₄₂ was analysed by flow-cytometry.

3.2.4 Effects of MaR1 on microglia phenotype markers

In **Paper IV**, to analyse the effects of MaR1 on microglial phenotype, the CHME3 cells were seeded into 6-well plates and incubated with 0 - 1 μ M MaR1 for 6 h, at which time point the cells were harvested for flow-cytometry analysis.

3.3 ANALYTICAL TECHNIQUES

3.3.1 Enzyme immunoassay (EIA)

Enzyme immunoassays (EIAs) were used through **Paper I-III**. The assays included LXA₄ EIA kit (Oxford Biochemical Research, MI, USA), RvD1 EIA kit and LTB₄ EIA kit (both from Cayman Chemical, MI, USA). These EIA assays are based on competition between the analyte in the sample and an enzyme-conjugated analyte, to bind to the limited amount of antibodies attached to the plate well. Thus, the amount of analyte in the sample is inversely correlated to the optical signals detected. Compared to LC-MS-MS, the advantage of using EIA assays is that many samples can be assessed simultaneously in one plate, making the comparison between the samples faster and less variable. On the other hand, the EIA assays may be less specific than LC-MS-MS, and provide less analyte information.

For lipid extraction in CSF and culture medium, samples were diluted and acidified to pH 3.5. The acidified samples were then added at 0.5 ml/min onto a C18 column, preconditioned with methanol and water. The C18 column was then washed by water and hexane. Lipids were eluted by methyl formate, and dried by nitrogen gas. The dried samples were resuspended in extraction buffer supplied by the LXA₄ EIA kit, for further analysis. Tissues

were homogenized and centrifuged. The supernatants were collected and processed as described above. All of the analyses were performed according to the manufacturers' instructions.

3.3.2 LC-MS-MS

LC-MS-MS method was used in **Paper I and IV** for analysis of lipid mediators (LMs) in postmortem tissues from the hippocampus and entorhinal cortex. Compared to EIA assays, LC-MS-MS has higher specificity and broader range of detection (several types of analytes can be assessed simultaneously in one sample). However, LC-MS-MS costs much more time than EIA assays, and the accuracy and variation between different assessments are much dependent on the equipment, experimental settings, and the strategy of data analysis [204].

For lipid extraction, tissues were gently dispersed by a glass dounce. Internal deuterium-labelled standards in ice-cold methanol were added into each sample, in order to facilitate quantification and sample recovery. The samples were kept at -20°C for 45 min for protein precipitation, and were then centrifuged (4°C for 10 min). Supernatants were collected and brought to less than 1 ml of methanol content by nitrogen gas. An automated extraction system (RapidTrace, Biotage, NC, USA) was used to extract the samples as in [128,205].

The LC-MS-MS system included a LC-20AD HPLC and a SIL-20AC auto-injector (Shimadzu, Kyoto, Japan), paired with a QTrap 6500 (ABSciex, CA, USA). LMs were eluted through an Eclipse Plus C18 column with a flow rate of 0.4 ml/min. The QTrap 6500 was operated in negative ionization mode. Scheduled multiple reaction monitoring (MRM) with a 90 s window was coupled with information-dependent acquisition (IDA) and an enhanced product ion scan (EPI) [128,205]. Each LM parameter was optimized individually, and was monitored by recently published criteria [128,205], including matching retention time to synthetic and authentic materials, and employing at least six diagnostic ions for each LM.

3.3.3 Multiplex cytokine assay

In **Paper I**, a human inflammatory cytokine assay (MesoScale Discovery, MD, USA) was used for analysis of the cytokine levels in the hippocampus. This multiplex assay is in a

sandwich immunoassay format similar to traditional enzyme-linked immunosorbent assay (ELISA), but can detect multiple analytes in one single assay, as the plate is pre-coated with several types of antibodies on separated spots in each well. Compared to traditional ELISAs that can only analyse one marker in one experiment, the multiplex assay makes it possible to assess more markers simultaneously, and use less samples. This advantage is valuable for optimized usage of samples that are in a limited quantity, *e.g.* human brain samples.

The cytokines/chemokine that were analysed include IFN- γ , IL-1 β , IL-12p70, IL-6, IL-8, IL-10 and TNF- α . Hippocampal tissues were homogenized by sonication. The homogenates were centrifuged at 20 000 x g for 15 min at 4°C, and the supernatants were collected for analysis. The MesoScale assay was performed according to the manufacturer's instruction.

3.3.4 Western blot

Western blot is a semi-quantitative antibody-based method to assess levels of proteins that are separated by electrophoresis. In **Paper I and II**, human and mouse hippocampal samples were analysed by western blot. Tissues were homogenized as in section 3.3.3. Each sample containing 40 μ g protein was boiled in Laemmli sample buffer at 95°C for 5min. The denatured proteins were separated by 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat milk for 30 min at room temperature (RT), and then incubated at 4°C overnight with primary antibodies. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at RT. Finally, the membranes were developed with electrochemiluminescence reagent, followed by detection with a CCD camera (Fuji Film, Tokyo, Japan). Analysis of the blots was carried out using Multi Gauge software (Fuji Film, Tokyo, Japan). All the blots were normalized to β -actin as the housekeeping protein.

3.3.5 Immunohistochemistry

Immunohistochemistry is a useful technique to study both morphological and quantitative aspects of a marker in tissue sections. So far, the distribution of resolution pathway markers in the brain is only partially described, and it was of interest to further analyse these in the human and mouse brain tissues. Moreover, when a marker is expressed by different types of

cells in the tissue, the immunohistochemical technique enables assessment of the expression in all cell types simultaneously, whereas the western blot technique will only analyse the total levels from all a mixture of cells.

3.3.5.1 Paraffin-embedded human brain tissue

Paraffin embedding allows long-term storage of samples without temperature control, and preserves better the morphological structures than frozen tissue. This is advantage that is taken into consideration when processing human postmortem samples, which are limited in source and quantity. However, paraffin embedding may influence the antigenicity. If a certain marker is undetectable, an antigen retrieval method can be applied. Another problem in the case of postmortem brain tissue from aged individuals, is the autofluorescence due to lipofuscin, which makes it difficult to distinguish the specific antibody staining from unspecific autofluorescence. Thus, peroxidase- or alkaline phosphatase (AP)-based immunohistochemical methods are commonly used for postmortem human brain tissue.

The paraffin-embedded tissue sections (**Paper I**) were deparaffinized, and endogenous peroxidase was blocked with 1% H₂O₂. After subsequent blocking with 5% serum at RT for 30 min, the sections were incubated overnight at 4°C with primary antibodies. After washing, the sections were incubated with biotin-conjugated secondary antibodies for 1.5 h at RT, followed by incubation with streptavidin-HRP complex for 30 min at RT. The staining was developed with 1 mg/ml diaminobenzidine (DAB) solution in the presence of 0.02% H₂O₂, and counterstained with cresyl violet.

Double immunostaining on paraffin-embedded tissue (**Paper I**) was performed as described above, except for an additional step of heat-induced antigen retrieval for the human leukocyte antigen type DR (HLA-DR) staining. The sections were incubated with BLOXALL (Vector Laboratories, CA, USA) to block endogenous peroxidase and AP. After serum blocking, the sections were incubated with primary antibodies including ALX/FPR2, ChemR23 and 15-LOX-2, mixed with antibodies to the astrocyte marker glial fibrillary acid protein (GFAP), or the microglia marker HLA-DR. After washing, a mixture of biotin- or AP- conjugated secondary antibodies was applied for 1.5 h at RT, followed by streptavidin-HRP complex

incubation for 30 min at RT. Peroxidase-based staining was developed with Nova Red, and AP-based staining was developed with Vector Blue (Vector Laboratories, CA, USA).

3.3.5.2 *Frozen mouse brain tissue*

Immunofluorescence is based on fluorophore-conjugated antibodies, and can be used in confocal laser microscopy or two-photon excitation microscopy. The major limitation of immunofluorescence is photobleaching due to photochemical destruction.

For immunofluorescent staining on frozen mouse tissues (**Paper II**), the sections were blocked with serum, and then incubated over night at 4°C with primary antibodies, followed by incubation with appropriate fluorophore-conjugated secondary antibodies for 1 hr at RT to visualize the staining.

For double labelling on frozen mouse tissues (**Paper II**), sections were incubated with a mixture of: *i*) neuronal marker NeuN and one of the antibodies ALX/FPR2, ChemR23, L12-LOX and 5-LOX, *ii*) GFAP or F4/80 mixed with 5-LOX, *iii*) L12-LOX and A β marker 4G8. A mixture of the appropriate secondary antibodies conjugated with red or green fluorophores was used to detect the staining.

3.3.6 Lactate dehydrogenase (LDH) and resazurin assays

LDH is a stable cytosolic enzyme that catalyses the conversion of NADH to NAD. When cell membrane permeability is disturbed due to cell death or injury, LDH will be released into extracellular space. Analysis of the enzymatic activity of LDH, released from damaged cells into the culture medium, serves as a measure of cell death. Resazurin is a blue chemical that can be converted into fluorescent resorufin by viable cells. Thus, the resazurin assay is used to assess cell viability in a cell culture. In **Paper IV**, we used both methods to assess the neuroprotective effects of SPMs on neuronal cells.

Neuronal cell death was assessed by LDH assay. Cell culture medium was transferred to a 96-well plate and incubated with LDH reagent for 30 min, and the absorbance were measured at 340 nm. The cells, which remain in the culture well, were assessed for cell viability by the

resazurin assay. The cells were incubated with resazurin salt at the final concentration of 0.01% for 2 h at 37°C, and the fluorescence intensity was analysed at 590 nm.

3.3.7 Flow-cytometry

Flow-cytometry is a laser-based technique widely used in cellular studies. In **Paper IV**, the phagocytosis of A β and the analysis of microglial phenotype were assessed by flow-cytometry. The fixed cells were washed and centrifuged at 400 x g for 10 min, and then resuspended with 1% bovine serum albumin (BSA). Cell suspensions were incubated for 45 min on ice with fluorophore-conjugated antibodies. The cells were then washed and resuspended in 200 ml 1% BSA, and analysed in a FACSCalibur cytometer (Becton Dickinson, NJ, USA). The proportion of microglia positive for each cell surface marker was assessed by determining the percentage of cells showing a signal in the proper channel exceeding the signal of the isotype control.

3.4 STATISTICS

All statistical analyses were performed with SPSS software (IBM Corporation, NY, USA). Analysis of covariance (ANCOVA) was used in the analysis of EIA data on CSF samples in **Paper I**, using age as a covariance, because age is different among AD, MCI and SCI groups. The Mann-Whitney *U*-test or Student's *t*-test was used to compare the group-wise differences in **Paper I, III and IV**, depending on whether the data were normally distributed. Two-way analysis of variance (ANOVA) using univariate general linear model (GLM) was used in **Paper II**, considering age and strain as two dependent variables. To compare the treatment effects of n-3 fatty acids and placebo, Wilcoxon signed rank test for analysis of paired samples was used in **Paper III**. The Kruskal-Wallis test followed by Mann-Whitney test was used to compare the effects of SPMs on neuronal and microglial cells in **Paper IV**. All through the studies, $p < 0.05$ was considered as statistical significant.

4 ETHICAL ASPECTS

For **Paper I and IV**, the use of human CSF and postmortem brain samples has been approved by the ethical committee at Karolinska Institutet, the regional human ethics committee of the Stockholm County, and the Swedish Ministry of Health and Social Affairs. The ethical permit numbers are: 2011/680-31/1 for human CSF samples; Dnr 024/01 and 2011/962-31/1 for human postmortem brain tissue.

For **Paper II**, SAMP8 and SAMR1 mice were kept and sampled in University of Navarra, Spain. The use of SAM8 and SAMR1 mouse hippocampal tissue has been approved by the ethical committee at University of Navarra, in accordance with the laws of animal experiments, European Directive 86/609/EEC and the Spanish Real Decreto 1201/2005. The ethical permit number is: 004/08.

For **Paper III**, the clinical trial of OmegAD study has been approved by the ethical committee at Karolinska Institutet, the regional human ethics committee of the Stockholm County, and the Swedish Ministry of Health and Social Affairs. The ethical permit number is Dnr 291/00. All the AD patients and their caregivers gave written consent before being enrolled in the clinical trial.

5 RESULTS AND DISCUSSION

5.1 RESOLUTION OF INFLAMMATION IN AD PATIENTS - OBSERVATIONS AND IDEAS (PAPER I AND IV)

SPMs are the key players in resolution of inflammation. To determine the resolution status in the AD brain, SPMs are of great importance to study. In **Paper I and IV**, we investigated the levels of SPMs in the postmortem brain of AD patients in comparison with age-matched controls. In **Paper I**, lower levels of LXA₄ and MaR1 were found in the hippocampus of AD patients, whereas the levels of RvD1 did not show statistical differences compared to controls. In **Paper IV**, we further investigated a more complete profile of SPMs in the human entorhinal cortex, and found that the levels of RvD5, MaR1 and NPD1 were reduced in AD, whereas other SPMs were not. Rather, the collective levels of all RvEs were increased in the entorhinal cortex of AD patients. Together with data from a previous report [52], it seems that the levels of LXA₄, MaR1 and NPD1 are decreased in the hippocampus, and that the levels of RvD5, MaR1 and NPD1 are decreased in the entorhinal cortex in AD patients, whereas the combined levels of E series resolvins are increased. These findings provide a characterization of the resolution pathway in the AD brain, but also provoke further questions, which could be partially answered in this thesis.

5.1.1 Is there a resolution failure in the AD brain?

Although SPMs are key players in resolution, measurements of SPMs could merely tell one side of the balance between inflammation and resolution. In **Paper I**, we have thus analysed the cytokine profile in the hippocampus of the same groups of AD patients and healthy controls. Among the 7 cytokines analysed, the levels of IL-1 β , IL-6, IL-8, IL-12p70, IFN- γ and TNF- α , did not differ between AD and control group. However, the anti-inflammatory cytokine IL-10 was reduced in AD. As shown previously, the regulation of IL-10 expression is an important functional pathway to achieve resolution [206,207], and decreased levels of IL-10 may represent a net effect of the balance between inflammation and resolution. Moreover, although we could not confirm increased levels of pro-inflammatory cytokines, shown in some earlier reports, we did observe increased levels of HLA-DR in glial cells

(Figure 6), providing evidence of elevated pro-inflammatory actions other than cytokines. Furthermore, the hippocampal levels of peroxisome proliferator-activated receptor- γ (PPAR- γ) were higher in AD patients. PPAR- γ has been shown to mediate LXA₄-ALX/FPR2 signalling, and activation of PPAR- γ increased LXA₄ production in an experimental stroke model [176]. A similar mediator function of PPAR- γ was also found in NPD1 signalling [208]. Our findings of increased PPAR- γ levels in AD suggest a compensatory response to the reduced LXA₄ production and insufficient pro-resolving signalling.

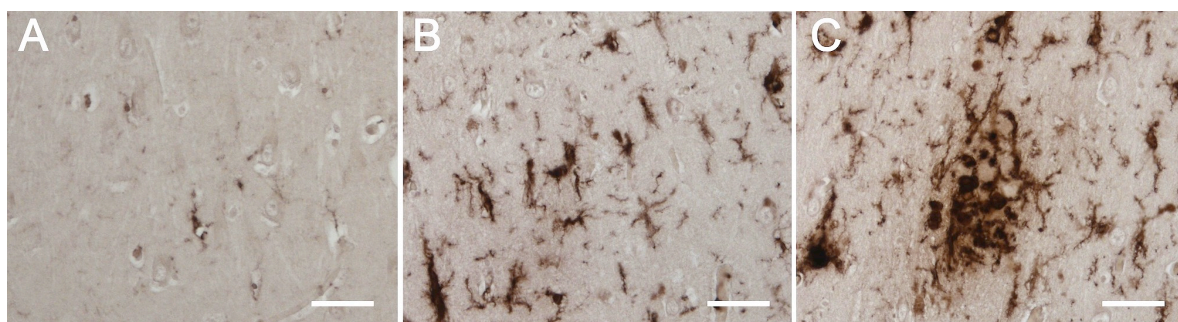


Figure 6. Immunohistochemistry of HLA-DR staining in human hippocampus. (A) control brain; (B) AD brain; (C) clustered staining in a plaque of AD brain. Scale bar = 50 μ m.

5.1.2 Why is there a failure of resolution?

Reduced SPMs can be the direct cause of resolution failure. While it was demonstrated that the relative percentage of AA and DHA in total FAs was reduced in AD [182], many other aspects need to be considered. For example, which cell types produce SPMs in the brain? In **Paper I**, we reported the existence of 15-LOX-2 in microglia and astrocytes. Unlike 15-LOX-1 that catalyses the conversion of AA to both 15(S)-p-HETE and 12(S)-p-HETE, 15-LOX-2 PPAR- γ catalyses the conversion of AA to 15(S)-p-HETE [209], indicating its involvement in LX synthesis [210,211]. Previous reports have shown that 15-LOX-1 and 5-LOX are present in glia and neurons [212,213], and that COX-2 is only expressed by neurons [214]. Since AD is a neurodegenerative disease, could it be the cell loss that causes reduced SPMs? As shown by another study, loss of neurons in the AD brain seems not able to cause the severe loss of NPD1 [52]. And there is a significant gliosis in the AD brain [215], so loss of glia cells are also excluded for a reason of less SPMs. Is it caused by the famous star A β ? We have recently shown that A β ₄₂ did not reduce the production of LXA₄ and RvD1 from microglia [216]. The answer may lie somewhere else.

Surprisingly, all the protein levels of enzymes involved in SPM synthesis were found to be increased in the AD hippocampus ([212-214] and **Paper I**). While reduced levels of DHA in the AD brain [52] may be a possible explanation of lower derivatives RvDs, NPD1 and MaRs, the decrease in LXA₄ remains unanswered. As shown in **Paper I and IV**, levels of the AA derivatives PG_s, TxB₂ and HETEs, were higher in the AD brain compared to controls. Thus, it seems that the AA cascade is producing less LXA₄, but more of pro-inflammatory products. Indeed, AA metabolism is up-regulated in the AD brain, as shown by injection of radio-labelled AA in AD patients followed by PET imaging [217]. These findings suggest that a functional abnormality of SPM enzymes may be the cause for the reduction in LXA₄ and other SPMs. It is interesting to note that the enzyme 15-LOX-2 is decreased, or even not expressed, in cancer [218-220], a disease with uncontrolled cell growth, compared to AD with uncontrolled cell degeneration. Moreover, it has been shown that phosphorylation of 5-LOX determines the production of 15-epi-LXA₄ or LTB₄ [221-223]. Thus, the exact role of LOXs in physiology and different diseases needs further research.

Pro-resolving signalling depends also on the receptors for SPMs. ALX/FPR2 is the receptor for both LXA₄ and RvD1 [123,131], as well as for A β [36,38-40]. In **Paper I**, we show that both neurons (CA2-4) and glia have staining for ALX/FPR2. The staining for ALX/FPR2 was higher in glial cells in AD brains compared to controls, but there was no difference in neuronal staining. Since LXA₄ is decreased and A β is increased in AD, it seems that there is an increase in detrimental A β -ALX/FPR2 signalling, and a decrease in beneficial LXA₄-ALX/FPR2 signalling, in glia and neurons of the AD brain.

5.1.3 Is there any clinical relevance?

Since postmortem examination is based on analysis of samples after death, a time point that usually represents the end stage of AD, it is difficult to tell how resolution markers are correlated to aetiology, clinical symptoms or pathological severity (all AD brains in **Paper I and IV** ranked Braak stage V-VI). However, it has been shown that in transgenic AD mice, LXA₄ treatment improved cognition, reduced AD-like pathology, and rescued from synaptic loss, via suppressed inflammation and enhanced resolution [183,184]. Moreover, LXA₄ and NPD1 provided pro-resolving effects and neuronal protection against A β toxicity in vitro

[52,208,224]. These studies provide substantial evidence that a lack of SPMs may be associated with less protection against AD, and may be a trigger or promoter for AD.

In **Paper I**, we also analysed CSF samples from AD and MCI patients, as well as from SCI subjects, using EIA kits for LXA₄ and RvD1. The levels of LXA₄ were lower in AD patients compared to those in MCI and SCI subjects, and there was no difference between MCI and SCI subjects. The levels of RvD1 in CSF did not differ among the groups. These findings confirmed the resolution failure as shown in the brain. Notably, there was a positive correlation between the levels in CSF of these two SPMs and the MMSE scores.

MCI and SCI are two special ‘control groups’. Mild cognitive impairment, MCI, is a term that defines a cognitive impairment that is worse than in normal aging, but not yet fulfils a diagnosis of dementia [225]. AD is the most common cause of MCI, or in other words, many MCI patients may convert to AD, in which case it is called MCI due to AD [225]. However, many other diseases, e.g. vascular dementia or Lewy body dementia, may also present as MCI at an early stage. Subjective cognitive impairment, SCI, is a term that is defined as self-noticed cognitive impairment that is severe enough for the patient to see a doctor, but the self-noticed impairment cannot be confirmed by the currently available cognitive tests, i.e. there is no objective proof of cognitive impairment [226]. However, this does not necessarily mean that the person has a completely healthy cognitive status. The symptoms may not be detected simply because of current methodological limits. Some of the cases with SCI may in fact be ‘preclinical AD’, in which the cognitive symptoms are lacking, but the biomarkers are already altered [227]. It has been proposed that the conversion rate from SCI to MCI is around 6.7% per year [226]. Similarly to MCI, SCI is also a group of patients affected by different diseases, such as AD and depression [226].

Despite the complexity of MCI and SCI, the levels of LXA₄ in the CSF showed a cut-off between AD and MCI/SCI, suggesting a role of SPMs in the conversion to AD. Moreover, there was a trend of higher levels of LXA₄ in SCI, suggesting that SCI is an even earlier stage of dementia than MCI. Whether decreased levels of SPMs in CSF samples can serve as early diagnostic markers needs further investigation.

5.1.4 Are SPMs always good?

In **Paper IV**, we show that the levels of RvEs are elevated in the AD entorhinal cortex. Rather than simply explaining the phenomenon as a complementary effect to the reduction of other SPMs, a more challenging question could be placed: are SPMs always good? It was reported previously that RvE1 could promote the activation of mammalian target of rapamycin (mTOR) and ribosomal protein S6 kinase (p70S6K) [228], which are key players in tau phosphorylation [229]. Thus, the elevated RvEs could be suspected to have a role in tau pathology in AD. More research is needed to give an answer, such as whether RvE1 specifically activates the mTOR complex 1 (mTORC1)/p70s6K pathway, that is responsible for tau phosphorylation, or whether it activates both mTORC1 and 2, of which the latter is supposed to be a cell survival promoter [230].

5.2 RESOLUTION OF INFLAMMATION IN AGING - REASONING (PAPER II AND III)

As indicated earlier in the thesis, aging is the greatest risk factor for developing AD [57]. Following our studies to characterize the status of resolution in AD patients, we then tried to explore how resolution is modulated in successful aging and abnormal aging.

5.2.1 What can be learned from successful aging?

In **Paper II**, we found an increase in MHC-II, a marker for activated microglia, with age in the mouse model of successful aging, SAMR1 mice. This is in line with the immunosenescence theory, that the macrophages become senescent with age, and increasingly display a more pro-inflammatory phenotype [65]. As aging is a chronic process with increased inflammation even in healthy conditions [65,66], it is important to notice the other end of the scale, the resolution of inflammation. In the SAMR1 mice, the LXA₄ and RvD1 levels were not increased or decreased with age, but their receptor, ALX/FPR2 was elevated with age. Thus, the pro-resolving signalling seemed to be enhanced through the receptor. Increased levels of ALX/FPR2 have been observed also after acute inflammatory conditions. For example, ALX/FPR2 expression was induced in horse tendons after acute injury, or after stimulation with IL-1 β or PGE₂, suggesting an endogenous program to resolve

inflammation [231]. However, a chronic increase in ALX/FPR2 may bring risks. As it is known that A β is also using ALX/FPR2 as a receptor to transduce pro-inflammatory signals [38-40], the increase in ALX/FPR2 levels with age is potentially creating an increased risk of A β -dependent toxicity in the brain. Importantly, this finding also indicated that aging and inflammation, rather than A β [216], may be the promoter for ALX/FPR2 expression. Moreover, chemerin, a chemoattractant molecule increased in multiple sclerosis [232], had a tendency towards reduction with age in the hippocampus of SAMR1 mice, indicating an attempt to lower the pro-inflammatory factors in a successful aging process.

To conclude, successful aging is achieved by balancing the inflammation and resolution, as shown in the SAMR1 model in **Paper II**. This idea agrees with the previous finding in humans, that healthy centenarians always present equal elevations of inflammatory and anti-inflammatory markers [67,68].

5.2.2 The unbalanced scale in abnormal aging

As we can learn from the SAMR1 mice and human centenarians, a balance between inflammation and resolution is essential to achieve successful aging. We did not observe a change in SPM levels in the old SAMP8 mice, neither with age nor compared to SAMR1 mice, but an excessive increase in inflammatory markers was obvious in the SAMP8 mice, as shown by previous studies [233,234]. Levels of MHC-II measured by western blot, and immunoreactivity of F4/80 shown by immunohistochemistry, were dramatically higher in the 9-month old SAMP8 mice, compared to 9-month old SAMR1 or 2-month old SAMP8 mice. In line with this, chemerin was increased with age in SAMP8 mice, compared to the trend of decrease in SAMR1 mice. We thus conclude that there was an elevated inflammation in the abnormal aging of SAMP8 mice, and that this increase was excessive in comparison with the normal aging in SAMR1 mice. On the other hand, resolution of inflammation in abnormal aging of SAMP8 mice remained at a similar level as that seen upon the normal aging of SAMR1 mice, as shown by equal levels of SPMs, as well as their receptor ALX/FPR2, in the two strains. Thus, excessive inflammation and insufficient resolution presented a disturbed balance in the SAMP8 mice.

5.2.3 From animal model to human translation

In **Paper III**, we analysed SPM release from PBMCs obtained from AD patients given placebo. After the 6 months placebo supplement, there was a drop in LXA₄ and RvD1 levels released from the PBMCs. Although a period of 6 months is quite short compared to the whole aging process, it is relatively long when compared to the 4 - 8 years survival time after a diagnosis of AD [57]. Thus, the reduction in LXA₄ and RvD1 release from PBMCs suggested a decreased ability of the body to produce SPMs with age in AD patients, providing partial support for the findings of decreased SPMs in **Paper I and IV**. The decreased secretion of SPMs upon age in humans (**Paper III**), and unchanged levels with age in the SAMP8 mice (**Paper II**) with aging may be simply reflecting the discrepancy between a human disease and an animal model. For example, it is well known that transgenic mice with mutant familial AD genes only present A β pathology, but rarely with tau pathology or neuronal loss [195]. However, it is also important to note that **Paper III** is an investigation of peripheral immune cells ex vivo, and whether the reduction seen in PBMCs reflects the change in the brain is unknown. Thus, whether decreased levels of SPMs in the AD brain and CSF represent early events or not, needs further investigation, but the SAMP8 and SAMR1 mice could still stand for good models to study the mechanism of balance between inflammation and resolution in abnormal and successful aging, respectively.

In the SAMP8 mice, we observed A β deposits in the hippocampus at 9 months, and this was accompanied by elevated p-tau levels compared to those at 2 months age. The simultaneous presence of AD-like pathologies and excessive inflammation in SAMP8 mice supports the relation between AD and inflammation. Interestingly, in the hippocampus of 9 months old SAMP8 mice, leukocyte type 12-LOX (L12-LOX, analogue of human 15-LOX-1) showed clustered immunostaining that was colocalized with A β deposits. Furthermore, there was a positive correlation between the levels of L12-LOX and p-tau levels in SAMP8, but not SAMR1 mice. Other studies showed that overexpression of L12-LOX increases the levels of A β [235], which may be produced at synapses [236]. Thus, the colocalization of L12-LOX and A β suggests a potential novel role of LOXs in the A β cascade, e.g. β - or γ -secretase activities. The correlation between L12-LOX and p-tau is in line with the previous report

showing that overexpression of L12-LOX increases p-tau levels in A β PP transgenic mice [237].

Overall, it seems that L12-LOX is a ‘bad guy’. Indeed, L12-LOX levels are increased in the AD brain [212]. However, there was no change with age in the SAMP8 mice, and there was even a decrease in L12-LOX levels compared to SAMR1 mice at 9 months. In contrast, L12-LOX levels were elevated with age in SAMR1 mice, the normal aging model. These observations raise questions regarding our theory, and to find an answer, we need to take a systematic overview. In the SAMR1 mice, while L12-LOX levels were increased with age, the levels of 5-LOX were decreased. Such a counter-regulation of L12-LOX and 5-LOX was proposed as an ‘anti-inflammatory’ activity in a previous report [238]. This active regulation of LOXs was not seen in the hippocampus of SAMP8 mice, in which both LOXs remained unchanged with age. Furthermore, the correlation between L12-LOX and p-tau was only observed in the SAMP8 strain, suggesting a functional alteration of this enzyme in this strain. These observations make further demands for research on the functional roles of LOXs in AD, as already discussed in **Section 5.1.2**.

5.3 EFFECTS OF N-3 PUFA SUPPLEMENTATION ON SPMS - PRACTICE (PAPER III)

SPMs are derivatives from PUFAs, especially the n-3 FAs DHA and EPA. The hypothesis of **Paper III** was that supplementation with DHA and EPA may result in an increase of their derivative SPMs. The study was based on a clinical trial, the OmegAD study (registered at clinicaltrials.gov as NCT00211159). It was a randomized, double-blind and placebo-controlled trial concluded by 174 AD patients. The primary outcome of the OmegAD study was that the cognitive decline did not differ between the n-3 FA- and placebo-supplemented group [112]. However, when analysing a sub-group of patients with MMSE > 27 prior to the trial, the cognitive decline was prevented by n-3 FA supplementation [112].

We obtained PBMCs from 15 patients, of whom 8 patients were supplemented with n-3 FAs, and 7 patients with placebo. Most of these 15 patients had relatively high MMSE scores before the trial, and hence the cognitive changes of these patients were in line with the

finding that cognitive decline was prevented by n-3 FA supplementation. The PBMCs were cultured *ex vivo* and exposed to 7 μ M A β ₄₀ peptide. Analysis of the PBMC culture medium, showed that levels of LXA₄ and RvD1 were reduced after 6 months placebo supplementation, and there was no change in the n-3 FA supplemented group. Collectively, the trend of changes in SPMs is in parallel with cognitive function, indicating a potential association. However, unlike the correlation between SPM levels and MMSE scores found in the CSF, statistical analysis did not show a correlation between the changes of SPM levels released from PBMCs, and the MMSE scores. Nevertheless, changes in SPMs were correlated to changes in the transthyretin (TTR) levels in plasma. TTR is a 55 kDa homotetramer, acting as a protein carrier in blood and CSF. It has been shown that TTR is present in senile plaques [239], and may play a beneficial role in AD through inhibition of A β toxicity [240-244]. Thus, the correlation between SPMs and TTR warranted further investigation regarding the relationship between SPMs and TTR.

Returning to the original hypothesis, supplementation with DHA and EPA may result in increased production of SPMs. The DHA and EPA levels were dramatically elevated in the plasma after 6 months of n-3 FA supplementation, and there was a small decrease in AA. These PUFAs were not changed in the placebo group. However, there was no change in the levels of RvD1 or LXA₄ after the n-3 supplementation. However, in the placebo group, there was a reduction in these SPMs, even though the PUFA levels in plasma were unchanged after the trial.

Indeed, supplementation with n-3 FAs has been shown to increase levels of NPD1, RvD1 and RvE1, in visceral adipose tissue of patients with severe obesity [245]. In healthy people, n-3 FA supplementation increased the plasma levels of RvE1, but not other SPMs [246]. Despite the different doses and duration of the supplementation, these complex results indicate that supplementation with n-3 FAs may not be sufficient to increase the SPM levels. This could be due to an altered incorporation of n-3 FAs into different tissues or a disturbed PUFA metabolism (e.g. abnormal functions of LOXs), especially under certain diseases, such as AD. For a sufficient supplementation with n-3 FAs to exert treatment effects, there are many questions placed beyond. For example, DHA transportation from the periphery to the brain

was reduced in ApoE4 transgenic mice [247]. It has not been clear until recently that DHA is transported across the blood brain barrier into the brain by Mfsd2a, a member of the major facilitator superfamily [248,249]. Whether Mfsd2a is altered in AD or in a person at risk to develop the disease, is unknown. Moreover, supplementation with n-3 FAs increases oxidative stress and shortens the lifespan of SAMP8 mice [250]. This further emphasizes the question whether PUFAs are metabolized into beneficial products in a proper way, and whether SPM enzymes are functioning correctly in certain populations with an abnormal aging process.

In summary, the efficacy of n-3 FA supplementation in AD patients is still far from a clear conclusion. A novel strategy based on SPMs instead of their precursors is appealing, from the perspective of a more direct intervention. For example, in a model for ischemic stroke, administration of NPD1 reduced total brain infarct volume by 87%, compared to DHA, 75% [174].

5.4 FUNCTIONAL ROLES OF SPMS IN THE BRAIN - LOOKING FORWARD (PAPER IV)

In order to provide more evidence regarding the potential SPM treatment effects in AD, we treated human neuronal cells and microglia with SPMs combined with different conditions in **Paper IV**.

5.4.1 Effects of SPMs on neurons - More than anti-inflammation

As described in **Paper I and II**, hippocampal neurons express ALX/FPR2, indicating a potential direct action of LXA₄ and RvD1 on neurons. In **Paper IV**, differentiated neuroblastoma cells were treated with SPMs (LXA₄, RvD1, NPD1 and MaR1) in the presence or absence of staurosporine. The SPMs did not only protect the neurons from staurosporine-induced apoptosis, but also enhanced cell survival at basal conditions. Staurosporine is a protein kinase inhibitor that is used in neurodegenerative models to induce apoptosis [251-253], probably via increasing oxidative stress [203]. The protective effects of SPMs against staurosporine are in line with previous reports on NPD1-induced

neuroprotection [254]. Despite the fact that the effects of SPMs in Study IV were obtained in vitro, it is of interest to note a neurotrophic effect of SPMs under basal conditions.

The direct effects of SPMs on neurons suggest that resolution, or pro-resolving mediators, may have other functions than to counteract/end inflammation. Besides to stop excessive and detrimental inflammatory signals, SPMs may also have follow-up effects by restoring homeostasis of the inflamed tissue, as evidenced previously by the long-term repair effects of DHA and NPD1 in a stroke model [174]. These results further support the idea that resolution is more than anti-inflammation [118].

5.4.2 Effects of SPMs on microglia - A focus on MaR1

A feature of pro-resolving activities is to enhance phagocytosis. We treated the human microglial cell line, CHME3, with four types of SPMs (LXA₄, RvD1, NPD1 and MaR1) in the presence of A β ₄₂ peptide. The microglial phagocytosis of A β ₄₂ peptide was increased by MaR1, but not by LXA₄, RvD1 or NPD1, all of which have been reported previously to increase phagocytosis in other cellular models [255,256]. Notably, LXA₄ and NPD1 were shown to enhance the phagocytosis of zymosan and latex beads by mouse macrophages in vitro [255], and RvD1 promoted phagocytosis of fibrillar A β ₄₂ by human macrophages, differentiated from PBMCs ex vivo [256]. The use of different cellular models and different targets for phagocytosis, may explain the discrepancy between our study and others. In continued studies on the CHME3 microglia, we focused on the effects of MaR1 on their phenotype. Under naïve conditions, MaR1 decreased the expression of the microglial surface marker CD33. CD33 expression was increased in the brain of AD patients, and inhibition of CD33 was found to increase A β clearance [257]. Thus, the enhanced A β ₄₂ uptake in CHME3 microglia may be mediated by reducing the CD33 expression. Furthermore, MaR1 reduced the pro-inflammatory markers CD80, CD86, CD11b and MHC-II, suggesting a suppression of M1 phenotype in CHME3 cells. However, the M2 anti-inflammatory markers CD163 and CD206 were not increased by MaR1 treatment, in contrast to a previous report on macrophages [143]. This discrepancy could be explained by the fact that CHME3 microglia express a high pro-inflammatory profile at basal condition [197], and thus MaR1 could only dampen the pro-inflammatory polarization, but not significantly enhance the anti-

inflammatory phenotype. The high pro-inflammatory polarization at basal conditions, and potential other genetic deficits, of the CHME3 cells may also be a reason for the lack of pro-phagocytic effects of LXA₄, RvD1 and NPD1.

Maresins are the most recently discovered SPMs, and further investigation is needed to characterize their roles in inflammation and disease. The evidence of reduced levels of MaR1 in AD brains, and the pro-resolving functions against A β and inflammation in vitro demonstrated by our studies provide fundamental new knowledge for further exploration of the role of MaR1 in AD.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

6.1 CONCLUDING REMARKS

The main aim of this thesis was to provide an overview of resolution of inflammation in AD. The results of our studies demonstrated a disturbed resolution pathway in AD patients, by analysis of brain and CSF samples, and functional cellular studies *ex vivo*. The thesis also describes basic characterization of the resolution pathway in the central nervous system, including the levels of SPMs, the distribution of SPM enzymes and receptors, and the levels of downstream effectors of SPMs. Moreover, mouse models of normal and abnormal aging were compared in the context of resolution. Finally, functions of four major SPMs on brain cells were analysed. The key findings can be concluded as:

- Levels of LXA₄, RvD5, NPD1 and MaR1 were reduced in the AD brain. LXA₄ levels were reduced in CSF samples of AD patients, and the CSF levels of LXA₄ and RvD1 were correlated with cognitive function.
- Distribution of SPM enzymes and receptors in the human brain were characterized, and an alteration of these factors were found in the AD brain.
- The release of SPMs from PBMCs of AD patients was reduced in a short aging period of 6 months, and the reduction was prevented by n-3 FA supplementation.
- Abnormal aging in mice was associated with insufficient pro-resolving signals, and altered resolution pathway factors were associated with AD-like pathologies.
- SPMs provided neuronal protection under basal and stimulated conditions. MaR1 enhanced microglial phagocytosis of A β ₄₂, and reduced microglial expression of pro-inflammatory markers.

6.2 FUTURE PERSPECTIVES

The discovery of SPMs is recent, and, probably, new SPMs will be found. Clearly, SPMs and the resolution of inflammation have emerging implications in many diseases including AD. However, there are many questions beyond establishing the full picture of SPMs and resolution in AD, such as:

- Is the deficit of resolution in AD a consequence, or a reason? We found a decreased ability to produce SPMs with age in AD patients, and there was a drop in CSF levels of SPMs between MCI/SCI and AD. It is still not clear how and why these changes occur, and whether leading or consequential.
- How do SPMs and resolution interact with the pathophysiology of AD? For example, since both LXA₄ and A β bind to ALX/FPR2, it will be of interest to establish whether the binding is competitive or not, and what is the consequential signalling on different cell types, *e.g.* neurons and glia.
- What is the specific role of each SPM? So far, most of the conclusions regarding SPMs have been collective from many different experimental settings that test one or two SPMs, and studies comparing the functions of SPMs are few, so far.
- Are SPMs always good? Based on current knowledge, SPMs have been proposed as beneficial, which may be true. However, because of their novelty, further research is necessary to confirm the safety. The RvE1-based drug RX-10001 has completed a Phase I trial, and RX-10045 has completed a Phase II trial on dry eye syndrome (registered at clinicaltrials.gov as NCT00941018 and NCT00799552, respectively). Concivably, these first SPM drugs will provide answers to some of the questions.

To picture the future much further ahead, despite the fact that it has not yet been clarified whether the disturbance of resolution is an aetiological cause, or a consequence of AD, SPMs represent a novel therapeutic strategy in AD, as well as many other inflammation-related diseases. Anti-inflammatory drugs did not favour the production of SPMs, but were rather ‘resolution toxic’ [146], indicating an additional reason for the failure of NSAIDs in clinical trials. PUFAs may have their own biological significance, and supplementation with PUFAs for human health overwhelmed the industry and research, as well as society. However, this should not hamper an investigation of novel and specific SPMs for the treatment in certain diseases associated with a disturbed resolution, such as AD.

Citing the words by Professor Bengt Samuelsson when talking about PGs, the future perspectives of SPMs and resolution of inflammation may be expected in a similar way: “It’s

a control system for the cells that participates in many biological functions. There are endless possibilities of manipulating this system in drug development.”

7 ACKNOWLEDGEMENTS

Life is about to thank. I am grateful for the capability and opportunity that the nature gives us to learn about nature. During the learning, there are so many thanks to:

Professor **Marianne Schultzberg**, my main supervisor, for your kind smile when we first met at the Arlanda Airport, and the nice bread and flowers for my first night in Stockholm. Thank you for your smart and patient guidance that raised me from a naïve researcher to grow-up. Thank you for your 360° support to me during the 5 years study and life in Sweden. You did not only teach me the correct way to learn the nature and science, but also taught me the good way to learn life and the world.

Dr. **Erik Hjorth**, my co-supervisor, for the friendship from colleague to supervisor. You are always there, with honest opinions and ideas, with calm attitude but warm heart, with objective comments to research and the news.

Professor **Ann-Charlotte Granholm**, my co-supervisor, for the storm of Lotta style that you brought from the United States. You always came to me with energy, inspiration and support. You made my knowledge and views much broader.

Professor **Gang Zhao**, my co-supervisor, for the enlightenment in the field of neuroscience. You are always supportive to my studies and career. Besides the clinical skills and knowledge, I learned so much about the ethics of being a clinician.

Associate Professor **Jie Zhu**, for introducing me to Karolinska Institutet and to my main supervisor. You guided me at the crossroad in my life, and helped me so much in my journey.

Mrs **Ping Zhang**, my mentor, for your nice assistance before my departure to Sweden, and for your kind help and advice during my study in Sweden.

All the present and former group members of the Schultzberg's research Family: **Heela Sarlus**, my dear sister, for growing up together, for the endless talk about souls and life, and for the collaborations of our projects, and so on... **Mingqin Zhu**, 'the little girl', for your kind heart and warm mind, for the nice talks in our mother tongue, for the way you always try to help. **Veronica Cortés-Toro**, for your so rigorous approach to the research work, for your great support and help in our collaborations. **Mircea Oprica**, for your always calm, relaxing and humorous attitude on everything, for your smart ideas during the discussions in lab meetings. **Lisa Schröder**, for your quick contribution to the group and good luck with your future studies. **Stefan Spulber**, **Catharina Lindberg** and **Åsa Forslin-Aronsson**, for all your excellent works in the lab previously, and for the nice time around Christmas tables and kayaking on the water. Thank you all, for as a group, and as a family gathered by research for human health!

All my collaborators during my PhD studies: the people from Karolinska Institutet, **Helga Eyjolfssdottir**, **Caroline Graff**, **Yvonne Freund-Levi**, **Lars-Olof Wahlund** and **Maria Eriksson** from Clinical Geriatrics division, and **Angel Cedazo-Minguez** from

Neurogeriatrics division, of our department, **Inger Nennesmo** from the Pathology Unit of Department of Laboratory Medicine of Karolinska Hospital Huddinge, **Inger Vedin** and **Jan Palmblad** from Department of Medicine (Huddinge); **Tommy Cederholm** from Department of Public Health and Caring Sciences, Uppsala University; from Spain, **Elena Puerta** from Department of Pharmacology, University of Navarra; from the United States, **Kumar Sambamurti** from Department of Neurosciences, Medical University of South Carolina, **Jonathan M. Fitzgerald**, **Romain A. Colas** and **Charles N. Serhan** from Brigham and Women's Hospital, Harvard Medical School of Harvard University.

Special thanks to those people who did not appear in the author lists of my publications: **Inga Volkmann** for the help with immunohistochemistry techniques, **Anna-Karin Lindström**, **Anna Sandebring** and **Mimi Westerlund** for the staff of Brain Bank at Karolinska Institutet. All the patients and other individuals who agreed to contribute to research. And for the unknown people who cleaned the microscopes after use, left the CCD camera open when seeing my booking, spared space on the membrane shaker...

All the senior scientists in the Department of NVS in NOVUM (those not mentioned elsewhere): **Abdul H. Mohammed**, **Agneta Nordberg**, **Amelia Marutle**, **Åke Seiger**, **Dag Aarsland**, **Elisabet Åkesson**, **Erik Sundström**, **Eiríkur Benediktsson**, **Helena Karlström**, **Homira Behbahani**, **Jan Johansson**, **Jinjing Pei**, **Lars Tjernberg**, **Susanne Frykman**, **Taher Darreh-Shori**, for your great works and ideas that made the NVS department outstanding and influenced us students positively!

The former and present Head of NVS department, Professor **Kerstin Tham** and Professor **Maria Eriksson** for creating a nice professional academic atmosphere in our department, and special thanks to Professor Eriksson for our scientific collaborations and happy talks outside the lab.

The Swedish Brain Power organization, especially Professor **Bengt Winblad** for creating such a nice network to gather the power of the most outstanding researchers in Sweden to fight against neurological diseases. Also **Gunilla Johansson** for the great administrative work in this organization.

The present and former colleagues in NVS: **Alina Codita**, **Antonio Piras**, **Azadeh Karami**, **Babak Hooshmand**, **Bo Li**, **Bo Zhang**, **Dan Wang**, **Daniela Enache**, **Elena Rodriguez-Vieitez**, **Eric Westman**, **Erica Lana**, **Erika Bereczki**, **Eva-Britt Samuelsson**, **Fuxiang Bao**, **Gabriela Spulber**, **Gefei Chen**, **Helen Poska**, **Hong Yu**, **Hongliang Zhang**, **Huei-Hsin Chiang**, **Jia Liu**, **Kai Niu**, **Kejia Zhang**, **Kevin Grimes**, **Lena Holmberg**, **Muhammad Al Mustafa Ismail**, **Nina Kronqvist**, **Ning Xu**, **Pavla Cermakova**, **Per Henrik Vincent**, **Qiupin Jia**, **Ruiqing Ni**, **Shouting Zhang**, **Torbjörn Persson**, **Xiangyu Zheng**, **Xiaoke Wang**, **Xiaozhen Li**, **Xijing Mao**, **Xingmei Zhang**, **Xu Wang**, **Walid Tajeddinn Abderhim**, **Yang Ruan**, **Zhi Tang**, **Zhongshi Xie**... all those who smiled!

All the administrative staff of NVS, especially **Maria Roos, Maggie Lukasiewicz, Annette Karlsson, Inger Juvas, and Anna Gustafsson**, and **Maria Ankarcrona**, Director of Doctoral Education at NVS, for arranging my whole doctoral study at NVS. Special thanks to **Ronnie Folkesson** about everyday life in the lab!

Dr. **Maoli Duan, Feng Gu** and **Yan Li** for the nice network of the Chinese Medical Association in Sweden.

The Chinese Embassy in Sweden, especially Mr. **Chunxiang Dou, Ning Zhang, Wei Wang, Rui Fan**, for all the support during my PhD studies in Sweden.

All my other Chinese friends in Sweden not mentioned above: **Professor Xiaokun Qi, Ruisheng Duan, Ronggui Li; Bin Zhao, Chi Ma, Hongqian Yang, Ting Zhuang, Meng Li, Jia Sun, Shaohua Xu, Jiaqi Huang, Xiaogai Li, Jie Su, Huijie Xiao, Jie Song, Xu He, Wangshu Jiang, Yajuan Wang, Zhiyang Song's family, Haixia Chen's family, Miao Zhao's family, Shanzheng Yang's family, Jiqing Zhu's family and Yutong Song's family.**

My parents, 我的父母, 感谢你们赋予我生命, 感谢你们养育我长大, 感谢你们教导我做人的道理, 感谢你们总是给予我无尽的爱和支持.....

My parents-in-law, 我的岳父岳母, 感谢你们无私的支持, 感谢你们善良的理解, 感谢你们坚定的信任, 感谢你们给予的一切.....

My daughter Zihan, thank you for coming into my life, for your sweet kisses and smiles, for your lovely touches and sounds and words, for your endless requests to tell you stories, for your cute blessing 'good luck, papa, I love you' everyday when I go to work... for everything you bring to the family, it is all with love... ..

My wife Yan, 感谢, ありがとう, 고마워 and thank you for everything! I tried to write something here to thank you, but found no words, in any language, were enough! It is all with love! 사랑해!

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